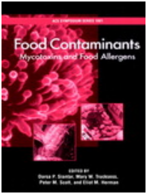

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Food Contaminants

Mycotoxins and Food Allergens

EDITED BY
Darsa P. Siantar, Mary W. Trucksess,
Peter M. Scott, and Eliot M. Herman

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ACS SYMPOSIUM SERIES **1001**

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Mycotoxins and Food Allergens

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Preface

This book includes about half the papers presented at the Symposia on Mycotoxins and Food Allergens, held during the 232nd American Chemical Society (ACS) National Meeting in San Francisco, California, September 10–14, 2006. The contrast between these two types of harmful substances with regard to their chemical nature, adverse effects, analysis, and control is clearly illustrated by these papers.

Mycotoxins are secondary fungal metabolites that are toxic to animals and plants. Several toxigenic fungi are pathogenic to important cereal crops. Fumonisins, trichothecenes, zearalenone, and patulin occur in food crops only as a result of a plant–fungus association; ochratoxin A is produced by species that are not involved in a plant–fungus association; and aflatoxins are formed by both mechanisms. The toxins themselves encompass a wide range of compounds, several of which have been shown to cause disease in domestic animals and, less frequently, in humans. Toxicology is represented in this book by papers on the measurement of aflatoxin-DNA adducts as biomarkers of long-term risk of disease in people, mechanism of fumonisin B_1 carcinogenesis, and enhancement of ochratoxin A toxicity by citrinin.

Approaches to controlling mycotoxins include attempts to prevent the formation of the toxins by the fungi, decreasing bioavailability of the toxins, and destroying toxins that have already been produced. The best solution to the problem of mycotoxins is to prevent their formation in crops in the first place, for example by competitive exclusion whereby non-aflatoxigenic strains of *Aspergillus flavus* or *A. parasiticus* are introduced onto the crops. Biotechnology is helping to provide protection of corn plants (Bt corn) against corn borer damage, thus reducing mycotoxin contamination. One potentially important method of controlling toxicity to animals is to reduce the bioavailability of the toxins, by adding sequestering agents such as silicate minerals and yeast-derived products to the feed.

Monitoring of the occurrence and levels of mycotoxins in commodities and foods is necessary to divert contaminated materials from human and animal food supplies and is important to estimate human exposure. Methods of analysis for mycotoxins include use of immunoaffinity columns, multifunctional mixed bed immunoaffinity columns, molecularly imprinted polymers, liquid chromatography–tandem mass spectrometry, and qualitative and quantitative lateral flow methods. Examples of application are methods of analysis for trichothecenes in cereal products, fumonisin B_1 in botanical roots, aflatoxin M_1 in milk, aflatoxins in peanuts, and ochratoxin A in wine.

Food allergenicity is increasingly in the public spotlight. News articles highlight the continuing growth in the numbers of food allergic people, along with the increased risk to those people as a result of broader and more intense sensitization. The underlying reason for the increasing rates of food allergy is a subject of debate and continuing research. It is clear that the causes are in large part environmental, which likely includes the exposure and lack of exposure to microorganisms, parasites, and immune challenges that separate the modern post-industrial lifestyle from that of our ancestors. The diets in the industrialized West, with its high food allergy rates, expose people to a diversity of food types never before included in the human diet. How an antiseptic environment, food diversity, lack of exposure to immune challenges, and even antibiotics may have a synergism to produce higher rates of food allergenic people still needs much research. The public concern about the rising food allergy rates has been addressed by increased government regulation and intervention both in the United States and in many European countries.

There are two main ways to address food allergy (i.e., medical treatment and prevention). Medical treatment is an active area of research and while there are promising developments the actual treatment of food allergy today has relatively little impact in managing food allergy. The use of epinephrine as a post-exposure treatment for serious food allergies has had a great impact in preventing anaphylaxis deaths following accidental exposure to food allergens. But the primary means to prevent exposure is variations on the theme of avoidance. Consumers and producers

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work together to provide avoidance mechanisms. For the consumer, vigilance on food items is essential, and for industry a wide variety of controls, including production and product line testing as well as labeling, are used to assist the consumer in making accurate avoidance choices. In the United States, the Food Allergen Labeling and Consumer Protection Act of 2004 came into force of law in 2006, providing for plain language labeling of eight major food allergens.

The food allergy papers in this book represent a broad spectrum of the research being done to support avoidance of food allergens. There are important issues of detection and the threshold of that detection. Modern techniques of mass spectroscopy and immunology are highlighted. Computer analysis can attempt to predict whether a new protein might be a food allergen. Such predictions will be useful, especially as genetic modification of food crops continues, to ensure those modifications do not introduce new food allergens. Technology is emerging to approach avoidance from the perspective of avoiding the allergen but not the food itself. Mutants and transgenics can be employed to create low allergen-content versions of otherwise allergenic foods. Whether this will actually mitigate the development of sensitization and manifestation of response in those people already sensitized remains to be determined. While it would have been wonderful for this book and the symposium to have announced the end of food allergies, this unfortunately will take more research and more years. However, with the broad application of avoidance techniques through analysis, labeling, medical testing, and possibly food modification, the rise in food allergy rates can hopefully be slowed and perhaps even reversed.

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Chapter 1

Natural Products as Tools for Chemogenomic Analysis of Mycotoxin Biosynthesis and Fungal Stress–Response Systems

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Certain phenolic compounds with antioxidant properties inhibit aflatoxin biosynthesis in the fungus *Aspergillus flavus*, without affecting growth. Similarly, some of the same phenolics also inhibit biosynthesis of ochratoxin by *A. alliaceous*. Exposing *A. flavus* to oxidative stress, such as hydrogen peroxide, enhances aflatoxin biosynthesis. Bioassays with gene-deletion mutants of *Saccharomyces cerevisiae*, as a model fungus, showed phenolics and reactive oxygen species modulated the antioxidative stress-response system. Caffeic acid was selected as a chemogenomic tool to monitor expression profiling using *A. flavus* microarrays. These profiles showed that treatment of the fungus with caffeic acid resulted in significant down-regulation of almost all genes in the aflatoxin biosynthetic gene cluster. However, there was little change in expression by *laeA* and *aflR*, known regulatory genes of aflatoxin synthesis. Alternatively, a number of peroxiredoxin genes most closely related to alkyl hydroperoxide reductases are dramatically up-regulated by the caffeic acid treatment. These enzymes are believed to reduce amounts of lipoperoxides. The lowering of such peroxides may reduce up-stream signaling from oxidative stress-response pathways that trigger aflatoxin biosynthesis. These results show antioxidative stress response genes are pivotal to modulating expression of the aflatoxin biosynthetic gene cluster.

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Extremely low regulatory levels (parts per billion, ppb) of aflatoxin contamination significantly affect the economic value of a number of agricultural products, especially with regard to exportability. Certain tree nuts, almonds, pistachios, and walnuts, are particularly affected by these restrictions on levels of aflatoxin contamination. Practically all tree nut production in the United States is in California. The overall value of these tree nuts approaches $4 billion, annually. Moreover, approximately 40-60% of these nuts are exported to countries with strict regulatory thresholds at 1-4 ppb (*1*). These same restrictions also affect the marketability of other US agricultural products, such as corn, peanuts and cottonseed. As such, methods are needed to reduce or eliminate aflatoxin contamination of agricultural products for not only food safety, but also marketability purposes.

Aflatoxigenesis and Oxidative Stress

Natural compounds that are components of agricultural products affected by aflatoxin contamination could play a role in preventing either fungal infection or aflatoxin biosynthesis. For example, a phenolic compound, gallic acid, discovered in the hydrolyzable tannins in walnut seedcoats was found to prevent aflatoxin biosynthesis by the fungus *Aspergillus flavus* (*2*). Bioassays involving gene deletion mutants of the yeast, *Saccharomyces cerevisiae*, as a model fungus, show that gallic acid acts as a potent antioxidant. These findings suggest there is an association between oxidative stress and aflatoxigenesis in *A. flavus* (*3*).

Following the discovery of the anti-aflatoxigenic activity of hydrolyzable tannins in the walnut seedcoat, an effort was made to determine if the other tree nuts also possessed anti-aflatoxigenic natural compounds. In fact, a number of highly active compounds were found in all tree nuts (Figure 1). As found in walnuts, hydrolyzable tannins in general are quite inhibitory. Pentagalloyl glucose had the greatest activity, inhibiting aflatoxin biosynthesis >99%, relative to controls. Caffeic acid, a phenolic compound found in pistachio hulls, had the second greatest inhibitory activity. The remaining compounds isolated with anti-aflatoxigenic activity were all phenolic compounds, the only exception being quinic acid. The discovery of these compounds having anti-aflatoxigenic activity further suggested that inhibiting oxidative stress in the fungus, in this case with anitoxidants, resulted in down-regulation of genes in the genetic pathways involved with aflatoxigenesis.

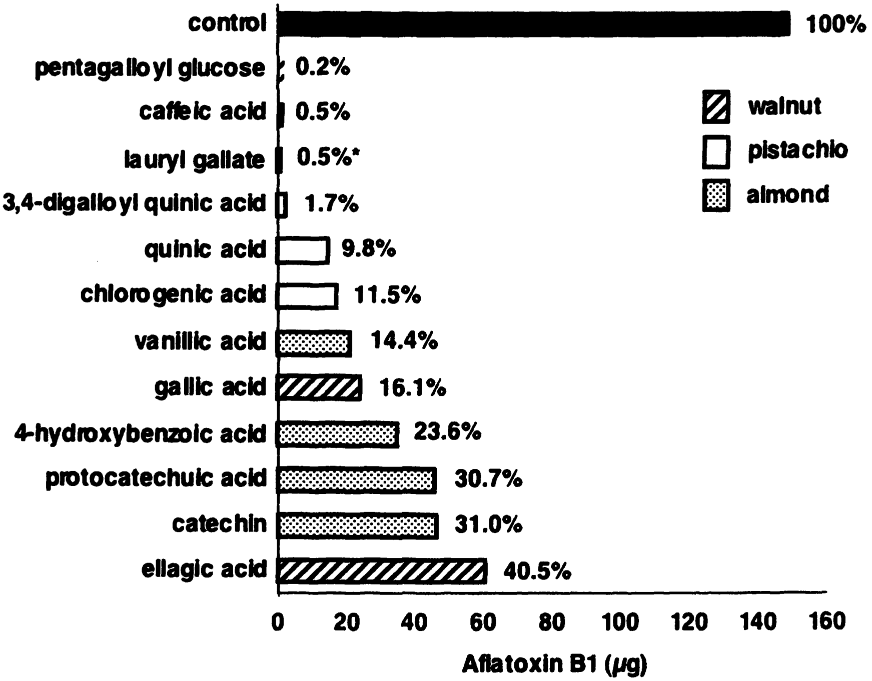

Figure 1. Comparative anti-aflatoxigenic capacity of tree nut constituents. All assays used 2 mM of test compound in media containing agar and ground pistachio kernels. Spores of Aspergillus flavus *NRRL3357 (≈200) were spotted in the center of the plates and incubated at 30 °C for 5 days.*
**model compound for anacardic acids in pistachio.*

Next, these tree nut constituents were tested against *A. alliaceus*, a fungus that produces a different mycotoxin, ochratoxin A. Like aflatoxin, the first steps in production of ochratoxin A also involve a polyketide synthase gene (*pks*). Hence, it seemed probable if expression of *pks* is somehow increased under oxidative stress conditions, perhaps antioxidants might also inhibit ochratoxin A

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biosynthesis. Interestingly, some of the tree nut constituents were quite anti-ochratoxigenic, but with some differences from those that were anti-aflatoxigenic. For example, vanillic acid had the greatest anti-ochratoxigenic activity at >99%. Caffeic acid was ≈94% inhibitory; whereas, gallic acid was only 40% inhibitory, and catechin, which was moderately anti-aflatoxigenic (Figure 1), actually stimulated ochratoxin A biosynthesis. Such results suggest there is some commonality in the association of oxidative stress and biosynthesis of certain oxygenated mycotoxins. But these preliminary results also indicate that chemical and genetic relationships between the antioxidative stress response and mycotoxigenesis may have certain independent and specific characteristics, depending on the mycotoxin and the fungal species.

Further support of a relationship between oxidative stress and aflatoxigenesis is prior work showing that oxidative stress by peroxides stimulates aflatoxin biosynthesis in another aflatoxigenic fungus, *A. parasiticus* (*4, 5*). When we expose *A. flavus* to peroxide stress, it also results in almost doubling aflatoxin production over a 9-day period (Figure 2). Although the experiments with *A. flavus* and *A. parasiticus* involved artificial induction of oxidative stress, natural oxidative stress to the fungus can result from host-plant production of toxic reactive oxygen species (ROS). Such ROS molecules include superoxide, hydrogen peroxide, hydroxyl radicals or organic peroxides. ROS can cause cell death as a result of lipid peroxidation, protein denaturation, damage to DNA, *etc.* ROS molecules can be generated as by-products of metabolic respiratory mechanisms in response to infecting microorganisms or abiotic stresses, such as drought (*6-8*). As aflatoxin biosynthesis is initiated by a polyketide synthase and intermediates in its synthesis are highly oxygenated, aflatoxigenesis itself may be a potential fungal response to sequester toxic ROS and thus is a means of protection from oxidative stress and ROS (*9*).

A number of cellular systems react to oxidative toxicity, some specifically to peroxides. Peroxiredoxins, peroxidases of the AhpC/TSA family, detoxify hydroperoxides by donation of electrons from NADPH (reduction) using thioredoxin/thiol-containing substances (*10*). Two transcription factors, Yap1p and Skn1p, have been identified that regulate the antioxidative stress-responses of yeast cells (*11*). Yap1p regulates amounts of GSH (reduced glutathione) *vs.* GSSG (oxidized glutathione) as a means of managing oxidative stress (*12, 13*). On the other hand, Skn1p regulates cellular thioredoxin/thiol homeostasis (*14*). Moreover, organic peroxides generated during fungal growth on lipid-containing substances (such as nut kernels) are likely sources of oxidative stress. The anti-aflatoxigenic activity of natural antioxidants, such as gallic acid, may be due to the attenuation of signals that trigger up-stream fungal oxidative stress responses. It is known that inhibition of aflatoxin production in *A. parasiticus* is through activation of an *hsf2*-like transcription factor regulating antioxidative enzyme production (*5*).

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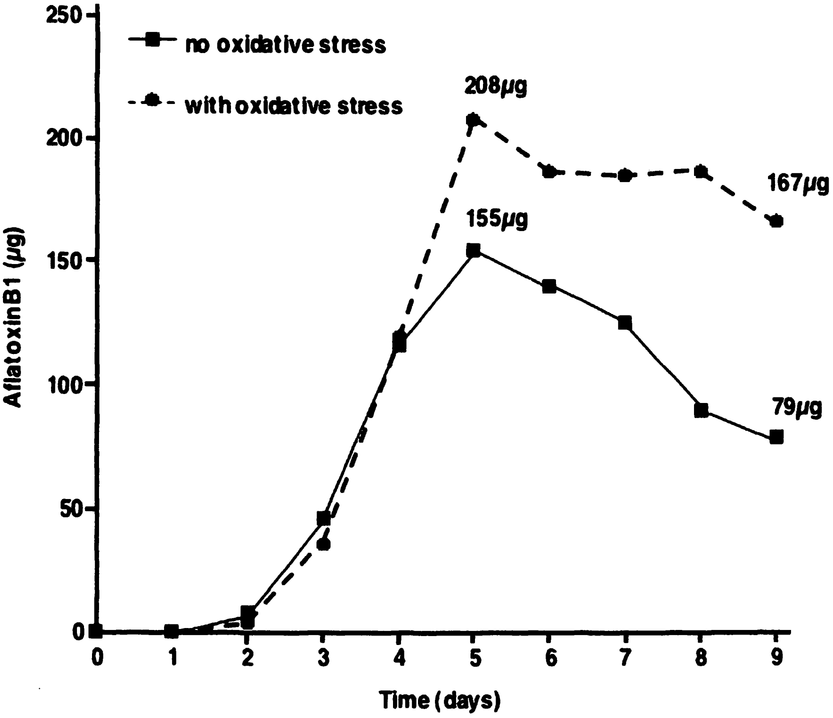

Figure 2. Aflatoxin production under oxidative stress. Oxidative stress was induced using 100 µM tert-*butyl hydroperoxide. Culture conditions as in Figure 1.*

Chemogenomics of Antioxidant Inhibition of Aflatoxigenesis

In view of findings of ourselves and others that oxidative stress induces aflatoxin biosynthesis and antioxidants inhibit its biosynthesis, we wanted to learn more about the functional genomic basis of these associations. Using yeast deletion mutants we had already identified a number of fungal genes that play a role in oxidative stress responses, including ones showing gallic acid acts as an antioxidant, reducing H_2O_2-based oxidative stress (*9*). Using an *A. flavus* Expressed Sequence Tag (EST) database (*15*) we were able to identify some orthologs of genes identified in our yeast studies in *A. flavus*. We hypothesized that some of these orthologs also played a role in the oxidative stress response of *A. flavus*, and thus might be involved in the regulation of aflatoxin biosynthesis.

In order to test our hypothesis we performed a comparison of the gene expression profiles of *A. flavus* under control conditions with that of fungi treated with one of the antioxidants already discovered that inhibits aflatoxigenesis. The effect of caffeic acid (12 mM) on aflatoxin production was evaluated over a 6-day period. Aflatoxin production in the treated cultures was reduced >95% compared to non-treated control plates (*e.g.,* day 6: 46 μg *vs.* 1.7 μg aflatoxin in culture with caffeic acid). However, growth and mass of the fungal mat was practically identical between the treatments, showing that caffeic acid did not inhibit fungal growth. These features, potent anti-aflatoxigenic activity with minimal antifungal activity, made this compound a particularly useful chemogenomic tool for examining global gene expression in relation to aflatoxin biosynthesis and antioxidant treatment. We thus used caffeic acid to perform a comparative microarray hybridization analysis to directly identify genes in the functional genomic response of *A. flavus* during suppression of aflatoxin biosynthesis.

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Gene Expression Profiles of *Aspergillus flavus* Treated with an Antioxidant

RNAs were isolated from control and caffeic acid treated fungi (4-day cultures) to compare quantitative changes in mRNAs between the treatments using microarray hybridizations. Changes in quantities of mRNAs were considered to reflect up-regulation (increase) or down-regulation (decrease) in expression of respective genes. Levels of change in gene expression were based on $\log_2$ transformed ratios of treated *vs.* control hybridizations, as follows: low ≥ -1.0 to ≤ 1.0); medium ≥ -2.0 to < -1.0, or >1.0 to ≤ 2.0; high < -2.0, or > 2.0. The more noteworthy results from this microarray analysis are summarized in Figure 3. A more detailed presentation of the microarray results is to be published elsewhere.

Down-regulation of Genes in the Aflatoxin Biosynthetic Cluster

Practically all mRNAs of genes in the aflatoxin biosynthetic cluster (*16*) showed significantly lower quantities ($\log_2$ ratios < -2.0) in the caffeic acid treatment. Examples from the microarray analysis included *verB*, a P450 monooxygenase involved in conversion of versicolorin B to A, and *omtB,* an *O*-methyltransferase converting demethylsterigmatocystin to sterigmatocystin (Figure 3). Declines in $\log_2$ ratios of other pathway genes in the microarray analysis ranged from low, –0.04, to high, –3.13, decreases. There was also a slight decline (-0.58) in expression of *aflJ*, a pathway transcription enhancer. We were unable to detect the pathway regulator gene, *aflR*, in the microarray analysis. Surprisingly, amounts of mRNA of a regulator gene for secondary metabolism, *laeA*, upstream from the cluster and reported to regulate aflatoxin biosynthesis (*17*), actually increased slightly (+0.23 microarray; +0.90 qRT-PCR) (Figure 3). This "up-regulation of *laeA* suggests this gene does not, alone, govern levels of aflatoxin biosynthesis.

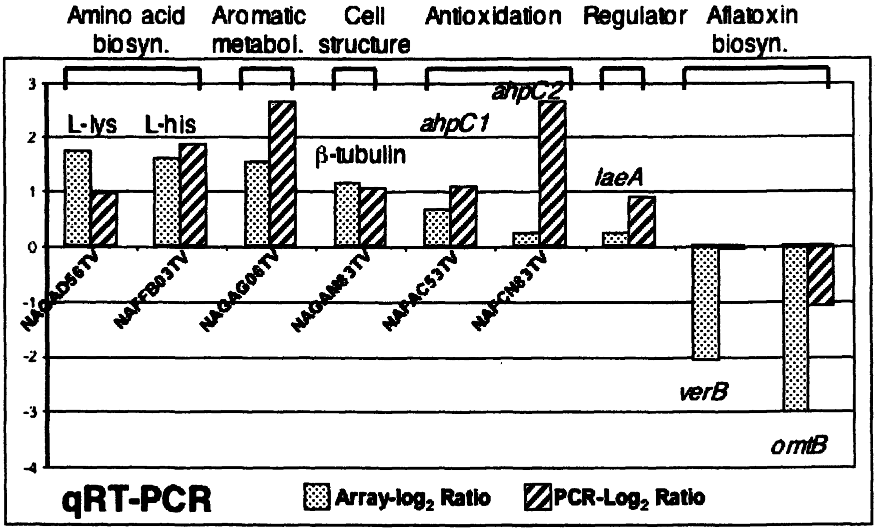

Figure 3. Summary of more notable results of gene expression analyses of Aspergillus flavus *treated with caffeic acid, compared to controls. Histograms represent level of* log_2 *transformed ratios (left axis) of differences in amounts of mRNAs (positive or negative) between treated and control fungi after a 4-day period. Differences are shown based on microarray analyses (dotted bars) and quantitative real-time PCR (qRT-PCR, hatched bars). Classes of genes are shown within brackets at the top of the graph and include genes involved in growth and aflatoxin biosynthesis.*

Other Down-regulated Transcripts

Thirty-six other transcripts, in addition to those in the aflatoxin biosynthetic pathway, showed some degree of down-regulation ($\log_2 < -1.0$) in the microarray analysis of the caffeic acid treatment. Most of these genes were involved in lipid metabolism, cell wall structure/integrity, molecular transport/pumping, and redox homeotasis. In addition hypothetical proteins and various genes were involved in a variety of functions, such as mitochondrial metabolism, DNA mismatch repair, *etc.*

Up-regulated Transcripts

The mRNAs of 28 genes showed some increases ($\log_2$ ratio > 1.0) resulting from the caffeic acid treatment. These genes were in 3 main categories, including various enzymes in amino acid metabolism, metabolism of aromatic compounds

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(Figure 3) and glutathione-*S*-transferases, in addition to a number of hypothetical proteins, an FAD-binding protein, a ribosomal protein, and others.

Up-regulation of Peroxiredoxin Genes

As stated above, a more thorough presentation of the comparative expression analyses from the caffeic acid treatment of *A. flavus* will be presented elsewhere. However, to summarize here some of the findings noted include only minor changes in genes involved in sugar utilization. But perhaps the most notable changes of all in our microarray analyses included up-regulation of four peroxiredoxin genes showing 38% to 50% sequence identity and 58% to 69% sequence homology, at the amino acid level, to Ahp1p (alkyl hydroperoxide reductase) of *S. cerevisiae*. Alkyl hydroperoxide reductases are important in detoxifying organic and lipid peroxides. Although expression levels of these peroxiredoxin genes were low in the microarray analysis, qRT-PCR (real-time quantitative reverse transcription-PCR) analysis showed there were dramatic increases in NAFAC53TV (*ahpC1*), NAFCN83TV (*ahpC2*) (Figure 3) and two others, NAGAD20TV and NAFDD05TV.

Discussion

We observed that aflatoxin production in *A. flavus* is greatly enhanced when incubated under oxidative stress induced by the organic peroxide, *tert*-butyl hydroperoxide (*t*-BuOOH). Also, we found that *A. flavus* already stressed by exposure to *t*-BuOOH and then treated with tannic acid, a commercial hydrolysable tannin containing only gallic acid moieties, reduced aflatoxin levels well below those observed without induced oxidative stress (unpublished results). Reverberi *et al.* (*5*) have shown that filtrates from the mushroom *Lentinula edodes* inhibit aflatoxin production by *A. parasiticus*, wherein the mode of action appeared to be delayed activation of *aflR*, the pathway regulator gene, and *norA*, an aflatoxin biosynthetic pathway gene. This filtrate is believed to contain a polysaccharide having lectin-like properties. Interestingly, treatment with this filtrate also triggers up-regulation for the antioxidant enzymes superoxide dismutase, catalase, and glutathione peroxidase, accompanied by activation of a yeast *hsf2*-like antioxidative transcription factor (*5*).

Based on microarray expression profiling, we were able to elucidate the functional genomic basis for the anti-aflatoxigenic activity of antioxidants. Expression was repressed for almost all genes in the aflatoxin biosynthetic gene cluster in *A. flavus* treated with caffeic acid. The mode of action of this anti-aflatoxigenic activity results from attenuation of the oxidative stress response to organic peroxides. Perhaps most notable in our analysis is induction of putative

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alkyl hydroperoxide reductases (AHP1) by caffeic acid. These AHP1 orthologs in *A. flavus* probably counteract upstream signals (*i.e.*, those involved with oxidative stress) that activate the aflatoxin biosynthetic pathway genes. As such, these enzymes play an important role in anti-aflatoxigenesis as well as tolerance to organic peroxides generated by fatty acid/lipid-rich substrates.

Metabolism of lipids/fatty acids stimulates aflatoxin production (*18*, *19*). Repression of lipases may be associated with the anti-aflatoxigenic activity resulting from treatment with caffeic acid. Caffeic acid may also inhibit enzymes involved in oxidation of cellular molecules (*e.g.*, lipids, proteins, DNA, *etc.*), which, in turn, may subdue signaling in pathways involved in oxidative stress responses. For example, methyl gallate inhibits aflatoxin biosynthesis (*2*) and also inhibits peroxide-induced oxidative stress in yeast (*3*). This compound was recently found to directly inhibit cyclooxygenase-2 (COX-2), an enzyme involved in lipid hydroperoxide formation (*20*). It has been known for some time that certain lipid peroxides from the host–plant are capable of modulating aflatoxin production (*21*). Thus disruption of normal lipoxygenase activity, either in host-plant tissues or in the fungus (*e.g.*, by inclusion of antioxidants in the culture medium), alters aflatoxin biosynthesis. Disruption of lipoperoxide production by the antioxidative activity of caffeic acid is probably the major biological component for its ability to inhibit aflatoxin biosynthesis.

The levels of caffeic acid we added to the culture medium resulted in almost complete repression of aflatoxin biosynthesis but did not affect fungal growth. The microarray profiles concurred with this observation in that genes involved in amino acid metabolic pathways and aromatic metabolism were up-regulated by the caffeic acid treatment. The up-regulation of these metabolic enzymes actually suggests that our caffeic acid treatments had a positive effect on fungal growth.

Conclusion

Oxidative stress triggers aflatoxin production in *A. flavus* and other aflatoxigenic species of aspergilli studied to date. Caffeic acid (and other phenolic anitoxidants) is a potent inhibitor of aflatoxigenesis and perhaps production of other mycotoxins (*e.g.*, ochratoxin A). Our functional genomic studies show that the mechanism for this antimycotoxigenic activity appears to involve attenuation of oxidative stress responses. The oxidative stress response pathways appear to provide upstream signals that induce aflatoxin production. Further functional genomic studies should enable elucidation of the genes involved in providing these signals that are linked to up-regulation of aflatoxin production.

In summary, merging of natural product chemistry with genomics has provided us with a powerful "chemogenomic" process. Use of such a chemogenomic approach will enable us to probe the functional genomic

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responses of fungi in a specific and measured fashion. This process will help in developing methods of preventing aflatoxin contamination,,either through chemical treatments, breeding of resistant crops or through some other form of genetic manipulation, as target genes involved in repressing aflatoxin production are identified.

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Chapter 2

Aflatoxin–DNA Adducts as Biomarkers of Cancer: Nature, Formation, Kinds of AF–DNA Adducts, Methodology, Effects, and Control

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Aflatoxins (AF) are potent carcinogens of foods, and the exposure of humans to them is continuous. Aflatoxin-DNA adduct's nature, chemical reactions, and molecular biology are of primary importance because they are the source of mutagenicity and risk of cancer in animals and humans. The measurement of aflatoxin-DNA adducts as biomarkers of long-term risk of disease in people, their proper assessment and the quantification of these active carcinogens is of great importance, because these adducts are directly related to the AF damaging effects and can explain the origin of the cancer under study. Relatively few biomarkers of long-term health have been even partially validated in experimental animals and in people. AF activates in the presence of the cytochrome P_{450}, as an unstable molecule called AF 8,9-epoxide; later this compound links mainly to the N^7 of the guanine nucleotide forming an adduct which is the active carcinogen itself and behaves as a biomarker, that is to say an objective measure of human exposure to environmental carcinogens. Adducts represent an integration of exposure, absorption, distribution, metabolism, DNA repair, and cell turnover, and thus provide a measure of biologically effective dose. The different issues presented in this chapter are: 1) Factors related to AFB-DNA adduct formation: age, cigarette smoking, alcohol drinking,

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organ susceptibility to form adducts (e.g., liver parenchymal cells, lung tissue, small intestine, tracheal explant cultures, placenta, bile radical intermediates, etc.), and temperature modulation of AFB_1 hepatic metabolism; 2) *In vitro* and *in vivo* studies of AFB_1-DNA adduct formation primarily at the N^7 position of guanine; 3) Formation of AFB_2 adducts; 4) Dietary AFB_1 exposure, development of human primary hepatocellular carcinoma (HCC) and mutations in the p53 tumor suppressor gene. The effect of diet in the adduct formation (choline-deficient/low methionine diet, food and caloric restriction, and the role of enzymes); 5) Routes of exposure for DNA adduct formation; 6) Vitamins; 7) Kinds of AF-DNA adducts (guanine, formamidopyrimidine), protein adducts (hemoglobin, albumin) and lysine, adenosine and cytosine AFB_1 adducts; 8) Methodology: a. reversed-phase high-pressure liquid chromatography (LC), b. a liquid chromatography electrospray tandem mass spectrometry (LC-ESI-MS/MS) method, c. polymerase chain reaction, d. radioactive methods, e. electron spin resonance (ESR) spectroscopy, f. enzyme-linked immunosorbent assay (ELISA) with monoclonal antibodies, g. indirect immunofluorescence analysis, and h. Ames test; 9) Effects; 10) Control, divided into sections: a) Natural repair rates of adduct removal, b) Induction of resistance to AFB_1, c) Detoxification enzymes (enzyme inhibition by *beta*-naphthoflavone), cytochrome P_{450} monooxygenases, d) Role of amino acid residues 209 and 365 of the P_{450} 2A5 in the metabolism and toxicity of AFB_1 using recombinant yeasts. e) Pre-exposure to AFM_1, f) Inhibition of AFB_1 lesions by different compounds (antioxidant ethoxyquin and enzymes), g) Protective chemical compounds and chemoprevention (dietary dithiolethione, nordihydroguaiaretic acid, butylated hydroxytoluene, and selenium), h) Natural nutrients from cruciferous vegetables (Brussels sprouts), Oltipraz with dithiolethiones, glucoraphanin and glucosinolate of broccoli sprouts and indole-3-carbinol); coumarin, ellagic acid from fruits and nuts; cafestol and kahweol from coffee beans; triterpenoid imidazole, monoterpenes; grapefruit juice; carotenoids; *Oldenlandia diffusa* and *Scutellaria barbata*; chlorophyllin; and probiotic bacteria.

The human and animal health implications that aflatoxins (AFs) have had in the last 46 years, since their discovery in 1960, have been due to their property of being powerful mutagens and carcinogens, with linkage to proteins and nucleic acids to form adducts that act as biomarkers of disease risk. In order to behave as mutagens and carcinogens, AFs have to be activated with the help of enzymes and cytochromes such as P_{450}, mainly in the liver, but also in other organs. The knowledge of AF-DNA adducts is basic to understanding the mutagenic mechanism of human cancer, among other diseases.

The information presented here is a summary of the knowledge of AF-DNA adducts until now, but the space to explain all the different scientific reports is not enough to give a complete overview, just an extract of the most important discoveries related to adducts as biomakers of disease.

AFs are secondary metabolites of the fungi *Aspergillus flavus, A. parasiticus* and *A. nomius,* with well known chemical properties (*1-3*). Aflatoxin B_1 (AFB_1) is the most toxic, mutagenic and carcinogenic aflatoxin and plays an important role in the etiology of human cancer (*4*). Although AFs were discovered more than 40 years ago, there is still considerable controversy surrounding their human health effects. Most countries have introduced legislation to control the level of AFs in food, but it is not known if these permitted levels still pose a significant cancer risk. Furthermore, it is unlikely that all the sources of human AF exposure have been discovered, and the liver is not the only, or indeed major, target organ for AF-induced cancer in man.

A main issue is how to assess the effects of AFs on long-term health. Bronson *et al.* (*5*) established that proper assessment of the damaging effects of AFs requires the measurement of biomarkers of long-term health in people consuming any particular phytonutrient. Relatively few biomarkers of long-term health have been even partially validated in experimental animals or people.

It is generally accepted that most chemical carcinogens induce cancer as initiators and act via a genotoxic mechanism (*6*). DNA is the key target of genotoxic carcinogens most of which require metabolic conversion into reactive forms (electrophiles), in order to undergo chemical reactions with DNA.

DNA adducts are reaction products with mutagenic propensity and specificity of the methods for their analysis. DNA adducts and DNA damage can arise indirectly by reactive oxygen species formed as consequence of chemical or radiation-induced perturbations in cellular metabolism (*7, 8*). Monitoring adduct levels in human tissue may provide information not only on carcinogen exposure but also on the relationship between infection with hepatitis B virus, dietary exposure to AFB_1, and liver cancer (*9*).

Factors Related to AFB-DNA Adduct Formation

AFs activate in the presence of the cytochrome P_{450}, forming an unstable molecule called AF 8,9-epoxide, which later links mainly to the N^7 of the guanine nucleotide forming an adduct that is the carcinogen itself and behaves as a biomarker, that is to say an objective measure of human exposure to environmental carcinogens. Questionnaire data or other environmental measurements are only complementary information. The adducts represent an integration of exposure, absorption, distribution, metabolism, DNA repair, and cell turnover, and thus provide a measure of biologically effective dose. Wild and Pisani (*10*) explain that the fact that DNA adducts are involved in the carcinogenic process means that their measure may be more relevant than other exposure measures based on ambient levels of a given carcinogen.

Risk Factors

The effects of multiple risk factors for hepatocellular carcinoma on formation of AFB_1-DNA adducts have been studied (*11*). Covalent binding of AFB_1 with hepatic DNA may be a critical step in hepatocarcinogenesis. The extent of the AFB_1 binding to DNA may depend on various endogenous factors and concurrent exposure to other environmental agents.

Age and the habits of cigarette smoking and alcohol drinking have a potential role in AFB_1-induced hepatocarcinogenesis and were also found to be associated with a higher percentage of AFB_1-N^7-guanine in total AFB_1 metabolite excretion, indicating an increased activation of AFB_1. No significant association with the AFB_1-DNA adduct level was observed for hepatitis B virus surface antigen carrier status, educational level, and ethnicity (*11*).

In relation to age and susceptibility of an organ to form adducts, Chelcheleh and Allameh (*12*) studied the ability of neonatal rat liver to metabolize [^{3}H]AFB_1 and compared it to that of the adult animal. They found a capability of neonatal rat liver to handle AFB_1, although the fate of large amounts of free (non-metabolized) AFB_1 deposited in the neonatal's liver is not well understood. AFB_1 is epoxidized more rapidly by the adult rat's liver and lungs 2 h after the toxin administration, compared with those of the neonatal rat (adult 30 pmol and neonatal 12 pmol AFB_1 bound/mg DNA). However, these differences were more pronounced in hepatic than in pulmonary tissues. These changes are related to the level of hepatic cytochrome P_{450}. The delayed cytochrome P_{450}-dependent AFB_1 activation in neonatal's liver provides time enough for de-epoxidation of slowly generated epoxide. The rate of AFB_1-epoxide formation at neonatal age was consistent with the activity of phase II metabolism of AFB_1 (glutathione conjugation). The hydrolysis of AFB_1-DNA adducts at a relatively higher rate by the neonatal liver may also contribute to the quick removal of the adducts (*12*). Shupe and Sell (*13*) found that low hepatic glutathione S-transferase (GST) and increased hepatic AFB_1-DNA adduction correlated with hepatocyte proliferation and contribute to increased tumorigenicity of AFB_1 in newborn and partially hepatectomized mice.

Bioactivation of AFB_1 in the Liver and Other Organs

There is an extrahepatic bioactivation of AFB_1 in fetal, infant, and adult rats. Tissue bound radioactivity was retained in the liver, in the mucosa, in some glands in the nose (fetal nasal olfactory tracheal mucosa, lateral nasal gland called Steno's gland), in the mucosa of the nasopharynx, trachea, bronchioles, mucosa of the glandular stomach, colon and caecum, but not in the small intestine, oesophagus or Harderian gland. GST activity catalysing the AFB_1 8,9-epoxide glutathione (GSH) conjugation is present in the nasal olfactory mucosa and liver at all pre-and post-natal ages examined (fetal, infant and adult rats) (*14*). *In vitro* experiments demonstrated that the nasal olfactory mucosa had a much higher capacity than the liver to form AFB_1 metabolites which are bound to DNA and protein (*14*). GST activity catalysing the AFB_1 8,9-epoxide GSH-conjugation was present in the nasal olfactory mucosa and liver at all pre- and post-natal ages examined. Several of the extrahepatic tissues able to bioactivate AFB_1 have been reported to be targets for the carcinogenicity of the substance. The extrahepatic carcinogenicity of AFB_1 is correlated to a local bioactivation in the sensitive tissues (*14*).

The liver is by far the main AF target organ and the induction of hepatocellular carcinoma from liver parenchymal cells in laboratory animals by AFB_1 is well documented (*15*). AFB_1-DNA binding was observed in both liver cell subpopulations and was 3- to 5-fold higher in parenchymal cells than in non-parenchymal cells. The major DNA adduct found in parenchymal cells at 1 h after AFB_1 administration was 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy-AFB_1 (AFB_1-gua), whereas later the persistent secondary adduct, AFB_1-formamidopyrimidine (AFB_1-FAPY), predominated. In contrast, AFB_1-gua was not observed at any time in DNA from non-parenchymal cells and the secondary adducts predominated throughout. Jennings *et al.* (*15*) discussed the susceptibility of different liver cell types to AFB_1-carcinogenesis and the possible roles of the major AFB_1-DNA adduct species. In contrast no tumors arising from the sinusoidal cell population have been reported after exposure to AFB_1. The apparent resistance of the latter cell type was investigated at the level of DNA adduct formation *in vivo* in male Sprague-Dawley rats (*15*).

AFs activate mainly in the liver, but also in other organs such as lungs (*12,16*), small intestine (*17*), nasal mucosa (*14*), etc. Donnelly *et al.* (*16*) investigated AFB_1 bioactivation, the role of enzymes, detoxification and pulmonary susceptibility to AFB_1 in human lung tissue obtained from patients undergoing clinically indicated lobectomy. Microsomal cytochrome P_{450} has a minor role in the bioactivation of AFB_1 in human lung. In addition to being a potent hepatocarcinogen, AFB_1 is a pulmonary carcinogen in experimental animals, and epidemiological studies have shown an association between AFB_1 exposure and lung cancer in humans. Co-oxidative bioactivation of AFB_1, in combination with the low conjugating activity displayed by human lung cytosolic GSTs, likely contributes to human pulmonary susceptibility to AFB_1 (*16*).

Intracellular AFB_1 adducts are formed in the small intestine, and this reflects, at least in part, the catalytic activity of cytochrome P_{450} (CYP) 3A enzymes. The small intestine may not play a significant role in the metabolism of AFB_1 (*17*). Because these AFB_1 adducts should ultimately pass in stool, enterocyte CYP3A may represent a regulatable barrier to dietary AFs (*18*).

Short-term tracheal explant cultures from the rabbit were used to study the metabolism of AFB_1 and to determine the cell types that are susceptible to damage by AFB_1 and their relative contents of mono-oxygenase enzymes (*19*). The conclusions of these studies are: a) rabbit tracheal explants are able to metabolize AFB_1; (b) the non-ciliated secretory cell population in this tissue is the target cell for cytotoxicity of this carcinogen; and (c) as is the case in the more distal airways, the non-ciliated epithelial cells appear to have a high content of components of the pulmonary cytochrome P_{450} monooxygenase system, which may be an important factor in the susceptibility of these cells and this region of the airways to suspected airborne carcinogens (*19*).

Hsieh and Hsieh (*20*) detected AFB_1-DNA adducts in human placenta (58%) and (9%) of adducts in cord blood, readily available specimens that respond to maternal environmental insult and are being used to investigate metabolism, bioactivation, and transplacental transfer of procarcinogens. Thus, monitoring adduct levels in human specimens may provide information not only on carcinogen exposure but also on the relationship among infection with hepatitis B/C virus, dietary exposure to AFB_1, and liver cancer.

Temperature Modulation

Studies on temperature modulation of AFB_1 hepatic metabolism, AFB_1 hepatic disposition, formation and persistence of DNA adducts studies in rainbow trout, *in vitro* and *in vivo*, have been carried out (*21*). The incidence of chemically induced tumors in fish increased with environmental temperature and genotoxicity. Adduction of $[^3H]AFB_1$ to hepatic DNA of 10 °C-acclimated fish was higher than that of 18 °C-acclimated fish after exposure and adducts were less persistent. When assayed at respective acclimation temperatures, hepatic cytosol from 18 °C fish produced more aflatoxicol (AFL), a detoxification product of AFB_1, than cytosol from 10 °C fish. This suggests the potential for complex interactions between temperature and metabolism of chemical carcinogens. This was probably explained by effects of temperature acclimation on the status of the membrane-bound cytochrome P_{450} system, affecting its competition with a detoxifying cytosolic enzyme. Such temperature-induced differences in microsomal cytochrome P_{450} isozymes and cytosolic dehydrogenase partially explain temperature-modulated AFB_1 genotoxicity (*21*). Temperature-modulated AFB_1 genotoxicity occurred via the mechanisms of hepatic disposition, formation and persistence of DNA adducts (*22*).

Dose Response Relationships

Quantitative carcinogenesis and dosimetry in rainbow trout for AFB_1 and AFL, two AFs that form the same DNA adduct, will be explained. Two exposure protocols were used to establish complete dose-response relationships for the hepatic carcinogenicity and DNA adduction *in vivo* of AFB_1 and AFL in rainbow trout. About 99% of AFL-DNA adducts produced *in vivo* were identical to those produced by AFB_1. Thus similar molecular dosimetry responses should be expected under all exposure protocols in which the two parent carcinogens do not exhibit differing toxicities to the target organ (*23*).

The formation of covalent AF-DNA adducts has been well studied in embryo and yearling stages of the rainbow trout (*Salmo gairdneri*) (*24*). The principal covalent product, identified after acid and enzymatic hydrolysis of isolated embryo DNA, was chromatographically identical to AFB_1-gua. The chemical transformation of this product in DNA produces AFB_1-FAPY and the metabolic activation of other AF metabolites, such as aflatoxin M_1 (AFM_1) and aflatoxin P_1 (AFP_1). The covalent modification of DNA was evident in eggs exposed to AFB_1 for a short time. The highest level of covalent products was present at approximately 24 h after exposure to 0.5 mg/kg AFB_1 (*24*). Ninety-six h later, this level had decreased slightly; however, the distribution of covalent adducts had changed: FAPY adducts now represented up to 50% of the hydrolyzed AF derivatives. A similar pattern of covalent AF derivatives was found in the liver DNA of yearling trout 10 h after the administration of 0.3 mg/kg AFB_1 (*24*).

Buss *et al.* (*25*) supported the fact that there is a linear dose-response relationship for DNA adducts in rat liver from chronic exposure to AFB_1. After chronic exposure to AFs, the level of DNA adducts did not increase significantly after 4 weeks, indicating a steady-state for adduct formation and removal had nearly been reached. At 8 weeks, the adduct levels were clearly proportional to the dose. At the high dose level, a near 50% tumor incidence would be expected in a 2-year bioassay with F-344 rats while the low dose used is within the range of estimated human dietary exposures to AFs in Western countries. There is a proportionality between exposure and steady-state DNA adduct level with respect to a linear extrapolation of the tumor risk to low dose (*25*).

Reactivity of AFB_1-DNA Adducts

AFB_1-epoxide reacts with base-paired DNA guanines in a sequence-specific manner. On the basis of a replication-block analysis, AFB_1-Cl_2 reacts with single-stranded DNA preferentially at inverted repeat sequences, which were suggested to be capable of forming intrastrand base-paired structures. Both AFB_1-epoxide and AFB_1-Cl_2 react with guanines in double-stranded DNA to induce similar sequence-specific, alkali-labile sites. AFB_1-Cl_2 was originally synthesized as an electronic analog for the putative AFB_1-epoxide, which has never been isolated due to its high reactivity. Reactivity with partial DNA duplexes as well as the use of single-strand specific chemical probes directly demonstrates that AFB_1-Cl_2, like AFB_1-epoxide, prefers base-paired guanines over non-base-paired guanines. DNA replication block patterns induced by AFB_1-epoxide are essentially similar to those induced by AFB_1-Cl_2.

Unexpectedly, and unlike other tested DNA lesions, Mn^{2+} does not appear to affect the template blocking properties of the adduct formed by AFB_1-Cl_2 or AFB_1-epoxide. The sites for replication stoppage as well as the lack of a Mn^{2+} effect on adducted templates have implications for the mechanisms of mutagenesis by activated AFB_1 (*26*).

DNA-damaging effects of genotoxins in a mixture and the modulation of covalent binding to DNA has been reported (*27*). Modulation of DNA adduct formation by pre-existing adducts was examined in synthetic oligonucleotides and genomic DNA (calf thymus); genotoxins studied were *N*-acetoxy-acetylaminofluorene, aminofluorene, AFB_1-8,9-epoxide, and dimethyl sulfate. The presence of the acetylaminofluorene adduct lowered the extent to which AFB_1-8,9-epoxide, but not dimethyl sulfate, reacted with target guanine. AFB_1 binding to DNA may be altered by conformational changes in the helix, due to the presence of a pre-existing acetylaminofluorene adduct, and there is absence of an effect by AF and confirmation of local denaturation of the oligomer helix by use of the chemical probes hydroxylamine and diethylpyrocarbonate. Nonetheless, the importance of changes in the nucleophilicity of neighboring nucleotides and local steric effects cannot be ruled out (*27*).

Formation of AFB_1-DNA Adducts

The *in vivo* formation of covalent AFB_1-DNA adducts within the ribosomal(r) RNA (rRNA) gene sequences of nuclear DNA has been studied in AFB_1-treated rats (*28*). Liver nuclear DNA, enriched in ribosomal DNA (rDNA) by one round of cesium salt density gradient centrifugation, was treated under buffered alkaline conditions to convert unstable AFB_1-N^7-guanine adducts to stable AFB_1-FAPY derivatives. rDNA contained 4- to 5-fold more AFB_1 residues than nuclear DNA, indicating that rDNA is preferentially accessible to carcinogen modification *in vivo*. While AFB_1 forms adducts with DNA principally at guanine residues, the guanine enrichment of rDNA was insufficient to explain the magnitude of observed preferential AFB_1 modification of rDNA. It is possible that rDNA regions are preferentially accessible to carcinogen modification because of the diffuse conformation maintained within transcribed genes. The quantitative description of carcinogen modification within a defined gene sequence may be useful in defining the precise relationships between covalent chemical-DNA interactions and the alterations in gene expression that result (*28*).

In relation to the formation of AFB_2 adducts there are some reports pointing out that AFB_1 2,3-oxide is a probable intermediate in the covalent binding of AFB_1 and AFB_2 to rat liver DNA and ribosomal RNA *in vivo* (*28*). Administration of tritiated $[^3H]AFB_2$ (2,3-dihydro-AFB_1)(AFB_2) to male rats resulted in levels of hepatic DNA- and ribosomal(r) RNA-AF adducts that were about 1% of those for rats given $[^3H]AFB_1$. The levels of hepatic protein-AF adducts were 35 to 70% as great for AFB_2-treated as compared to AFB_1-treated rats (*29*).

Troxel *et al.* (*30*) investigated hepatic AFB_1-DNA adduction in zebrafish (*Danio rerio*), the inductive response of cytochrome P_{450}1A (CYP1A), and the effect of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) on the metabolism of $[^3H]AFB_1$ following exposure to Aroclor 1254. The major $[^3H]AFB_1$ metabolites excreted in water over 24 h in the control group were AFL, AFL-glucuronide, and parent AFB_1 (*30*). In contrast, the predominant metabolites in the TCDD-pretreated group were AFL-M_1-glucuronide, AFL, AFM_1 plus AFL-M_1, AFL-glucuronide, and parent AFB_1. Surprisingly, hepatic AFB_1-DNA adduction was approximately fourfold higher in the TCDD treated group than in controls. Both control and TCDD treated zebrafish have high capacity to bioactivate AFM_1 to a reactive intermediate, such that secondary bioactivation of this genotoxic intermediate may be responsible for the increased DNA binding (*31*).

Denissenko *et al.* (*32*) have mapped total AFB_1 adducts in genomic DNA treated with AFB_1 8,9-epoxide and in hepatocytes exposed to AFB_1 activated by rat liver microsomes or human liver and enterocyte microsomal preparations. *In vitro* studies have shown that AFB_1 adducts form primarily at the N^7 position of guanine.The adduct patterns obtained with the epoxide and the different microsomal systems were virtually identical indicating that adducts form with a similar sequence-specificity *in vitro* and *in vivo*. The lesions were detected exclusively at guanines with a preference towards GpG and methylated CpG sequences (*32*).

Studies of Lee *et al.* (*33*) on liver revealed: a) no significant difference in the amount of adduct formed by DNA samples with or without integrated hepatitis B virus DNA; b) no difference in the amount of adduct formed with DNA from either tumorous or non-tumorous tissues from a given individual, and c) remarkable and reproducible differences in the capacity of DNA from different individuals to form *in vitro* adducts.

These last data contrast with the information given by Harrison *et al.* (*4*) who examined human DNA from a variety of tissues and organs to identify and quantify AF DNA-adducts. AFB_1-DNA adducts could be detected in formalin-fixed tissue from an acute poisoning incident in Southeast Asia (*4*). The authors have examined human colon and rectum DNA from normal and tumorous tissue obtained from cancer patients and colon, liver, pancreas, breast, and cervix DNA from autopsy specimens. AFB_1-DNA adducts were detected in all tissue types examined and ranged from 0-60 adducts/10^6 nucleotides, confirmed by LC analysis. Tumor tissues tended to have higher adduct levels than normal tissue from the same individual, and levels generally increased with patient age. In samples analyzed by LC, the adducts present had the chromatographic properties of 8,9-dihydro-8-(N^5-formyl)-2',5',6'-triamino-4'-oxo-(N^5-pyramidyl)-9-hydroxy–aflatoxin B_1, the ring-opened form of the AFB_1-guanine adduct (*4*).

Data of Lee *et al.* (*33*) also contrast with other reports (*34*), where hepatitis B antigen (HbsAg) carriers had a statistically significantly higher rate of detection of urinary AFB_1-N^7-guanine adducts than non-carriers.

The animal species also makes a difference in adduct formation. A comparison was made of AFB_1-DNA binding, adduct formation, AFB_1-DNA adduct repair, and their removal among three different species (rabbit, hamster and rat) that vary in amounts of cytochrome P_{450} in cultured mammalian tracheal epithelium (*35*). The binding of AFB_1 to DNA was highest in rabbit tracheal explants (78 pmol/mg DNA), followed by the hamster (28 pmol/mg DNA), with the rat (3 pmol/mg DNA) showing minimal AFB_1-DNA binding.

Organ Responses

Concerning the susceptibility to AFB_1 adduct formation, there are different organ responses. Liver and kidney might be the probable target organs for AFB_1 with the highest formation and persistence of DNA adducts (Choi *et al.* (*36*)).

The comparison of temporal patterns of exposure of DNA adduct formation and GST activity from liver and testes of rats fed with AFB_1 showed that the levels of testicular AFB_1-DNA adducts were 2.4 to 8.1 times lower than those of the liver. The testicular DNA adducts markedly decreased over time. In contrast, hepatic AFB_1-DNA adducts increased four-fold from 4 to 16 weeks of continuous treatment but increased at a much slower rate after intermittent exposure (*37*). In both the liver and testis, significant levels of AFB_1-DNA adducts persisted for at least 1 month after ending the treatment, suggesting that this type of lesion was poorly repaired. In control rats, the testis showed significantly higher GST activity than the liver. Tissue-specific differences such as germ-cell depletion and increased testicular detoxification may play an important role in the observed differential pattern of DNA adduct formation between the testis and liver (*37*).

Towner *et al.*(*38*) identified the trapped free bile radical intermediates such as a cytochrome P_{450} inhibitor dimethylaminoethyl-2,2-diphenylvalerate hydrochloride; and a Kupffer cell inactivator gadolinium chloride ($GdCl_3$) from the *in vivo* hepatic metabolism of AFB_1.

Dietary AFB_1 Exposure and Hepatocellular Carcinoma

Epidemiological evidence has been supporting a relationship between dietary AFB_1 exposure, development of human primary hepatocellular carcinoma (HCC) and mutations in the p53 tumor suppressor gene. However, the correlation between the observed p53 mutations, the AFB_1-DNA adducts and their activation pathways have not been elucidated (*39*). Development of relevant cellular *in vitro* models, taking into account species and tissue specificity, could significantly contribute to the knowledge of cytotoxicity and genotoxicity mechanisms of chemical procarcinogens, such as AFB_1, in humans (*39*). Therefore, the differential expression of specific cytochrome CYP_{450} genes in human hepatocytes can modulate the cytotoxicity, DNA adduct levels, and frequency of p53 mutations produced by AFB_1.

Effect of Diet on the Adduct Formation

The effect of diet on adduct formation is shown in several studies. Our first example, based on studies of Kimura *et al.* (*40*), compares the effects of diets (either AIN-76A or Purina Chow diet) on hepatic AFB_1-DNA binding and AFB_1-induced GST placental form positive hepatic foci have been examined in young male Fischer rats. Feeding of Purina Chow diet compared to AIN-76A diet inhibited the initiation phase whereas AIN-76A stimulated the promotion phase of AFB_1 hepatocarcinogenesis in rats by inhibiting AFB_1-DNA binding and increasing AFB_1-induced hepatic foci respectively (*40*).

There are also reports (*41*) about the influence of dietary fat on hepatic bioactivation of AFB_1 in rats. High-carbohydrate low-fat diets reduce microsome mediated epoxidation of AFB_1 to a larger extent than high-fat diets. In general, high fat diets increased cytochrome 1A1 and 2B1 activities relative to chow and high carbohydrate diet. This suggests greater detoxification of AFB_1, thus reducing the amount of AFB_1 available for hepatic macromolecular binding (*41*).

Schrager *et al.*(*42*) reported that nutritional modulation of male Fischer rats by a choline-deficient/low methionine- diet dramatically increased hepatocarcinogenesis and reduced time to first tumors induced by AFB_1. Total AF-DNA adduct levels in the choline-deficient animals were significantly increased during the multiple dose schedule. When total AF-DNA adduct levels were integrated over the 10 days dose period, a 41% increase in adduct burden was determined for the choline-deficient animals. This increase in DNA damage is consistent with the hypothesis that DNA damage is related to tumor outcome, although the biochemical basis for this effect still needs to be elucidated (*42*).

Metabolic activation of AFB_1 can be modulated by food. The AFB_1-N^7-Gua was predominantly formed in rat liver DNA; the formation of the open ring analogue of AFB_1-N^7-Gua, AFB_1-FAPY, was predominantly found in mouse liver DNA. In contrast to the *in vivo* results, the *in vitro* AFB_1-DNA adduct formation mediated by the microsomes of liver, kidney or lung gave opposite results. Food restriction induced hepatic GST activity, as measured by the formation of AFB_1-glutathione conjugates (AFB_1-SG), in rats, mice and in mouse kidney. Food restriction-induced GST activity assayed in an *in vitro* system, using $[^3H]AFB_1$-8,9-epoxide and glutathione as substrates, was also found when mouse kidney and lung cytosolic fractions were used (*43*) .

Dietary and caloric restrictions have been recommended by several authors (*43-45*) to reduce cancer risk. Sixty percent dietary restriction reduced AFB_1 metabolizing enzyme activity and decreased the AFB_1-DNA adduct formation in young rats treated with AFB_1. In aged *ad libitum* rats, the formation of AFB_1-DNA adducts diminished to the same level as that of dietary restricted groups and probably was due to the faster decline of drug metabolizing enzymes in aging rats. The stimulation of partial hepatectomy-induced liver regeneration by dietary restriction in aged animals may be attributed to the retardation of aging by the dietary restriction itself, and the retention of more active biochemical and enzymological functions in old dietary restriction-animals. A protective effect due to caloric restriction reduced the metabolic activation of AFB_1; this brought a decrease of AFB_1-DNA binding of more than 50% (*45*). Thus, the contributions of caloric restriction are the lower initial AFB_1.-DNA binding and less DNA damage, presumably by the fewer apurinic sites formed during the depurination process of AFB_1-DNA adducts and alteration of the formation of AFB_1-DNA adducts and AFB_1-SG conjugation. However, species and tissue specificities exist regarding the metabolic activation of AFB_1 (*45*).

Chou and Chen (*43*) examined the effects of food restriction on the metabolic activation of AFB_1 in rats and mice, which are AFB_1-sensitive and -resistant, respectively. In a comparison of food restriction and *ad libitum* food consumption treatments there was a reduction of metabolic activation of AFB_1 in both rats and mice, causing formation of hepatic AFB_1-DNA adducts to be 43% and 31% lower, respectively.

Role of Enzymes

Lipoxygenases are enzymes that play an important role in the bioactivation of AFB_1 in hepatic and extrahepatic tissues. The enzyme activities were evaluated by determining $[^3H]AFB_1$-DNA adduct formation. Lipoxygenase-catalyzed AFB_1 activation can occur at low AFB_1 concentrations. This may be important in view of human exposure to low AFB_1 concentrations and predominant lipoxygenase activity in human airway epithelial cells (*46*).

Hemoglobin did not contribute to AFB_1 activation attributed to guinea-pig tissue lipoxygenases. Lipoxygenases were largely responsible for the observed cytosolic activation of AFB_1. Rather, lipoxygenases and prostaglandin H synthase appear to be important bioactivation enzymes. Co-oxidative bioactivation of AFB_1, in combination with the low conjugating activity displayed by human lung cytosolic GSTs, likely contributes to human pulmonary susceptibility to AFB_1 (*16*).

Cytochromes P_{450} 3A4 and 3A5, the dominant drug-metabolizing enzymes in the human liver, share >85% primary amino acid sequence identity yet exhibit different selectivity toward AFB_1 biotransformation. A single P_{450} 3A5 substrate recognition sites domain (SRS-2) is capable of conferring the P_{450} 3A5 phenotype on P_{450} 3A4. Each substrate-P_{450} active site fit is indeed unique (*47*).

Reports about other important isozymes state that LMC2 is the most abundant hepatic cytochrome P_{450} found in sexually immature rainbow trout (*Onchorynchus mykiss*) and is also the isozyme that activates the carcinogen AFB_1. This P_{450} has been cloned, sequenced, and designated as CYP2K1. cDNA-expressed CYP2K1 protein is catalytically and immunologically identical to purified trout LMC2 and these two enzymes produce primarily the highly carcinogenic stereoisomeric *exo*-epoxide form of AFB_1 (*48*).

In the involvement of cytochrome P_{450}, GST, and epoxide hydrolase in the metabolism of AFB_1 and relevance to risk of human liver cancer, Guengerich *et al.(49)* found that AFB_1 is oxidized by human cytochrome P_{450} enzymes to several products. Only one of these, the 8,9-*exo*-epoxide, appears to be mutagenic and the others are detoxication products.

AFB_1 is detoxified to AFM_1 via cytochrome P_{450}-mediated AFB_1-4-hydroxylase. Genetic studies in mice have demonstrated that the expression of AFB_1-4-hydroxylase is regulated by the aryl hydrocarbon locus and suggested that different cytochrome P_{450} isozymes catalyze AFB_1-4-hydroxylase and aryl hydrocarbon hydroxylase activities. Faletto *et al.*(*50*) showed that cytochrome P3-$_{450}$ cDNA has the ability to metabolize AFB_1-4-hydroxylase, and there is a direct assignment of specific cytochrome P_{450} to an AFB_1 detoxification pathway. This finding may have relevance to the dietary modulation of AFB_1 hepatocarcinogenesis (*50*).

The aldehyde metabolite of AFB_1 may contribute to the cytotoxic effects. The AFB_1 aldehyde reductases, NADPH-dependent aldo-keto reductase superfamily AKR7A, metabolizes the AFB_1 dihydrodiol by forming AFB_1 dialcohol (*51*). Using recombinant human AKR7A3 and AFB_1 dihydrodiol at pH 7.4, the catalytic efficiency of this reaction equals or exceeds those reported for other enzymes, such as cytochrome P_{450s} and GSTs, known to metabolize AFB_1 *in vivo*. Depending on the extent of AFB_1 dihydrodiol formation, AKR7A may contribute to the protection against AFB_1-induced hepatotoxicity (*51*).

The mutagenic effect of AFB_1 results from hepatic bioactivation to AFB_1-*exo*-8,9-epoxide, in part catalysed by CYP3A5, an enzyme expressed polymorphically. The Gambia has a population exposed to high AF levels and the CYP3A5 polymorphism is associated with increased levels of the mutagenic AFB_1-*exo*-8,9-epoxide, particularly in individuals with low CYP3A4, and this may modulate individual risk of HCC (*52*).

There is contradictory information about the importance of the locus and the genes that are determinant to activate AFB_1; in this respect serious analysis (*53*) states that codon 249 of the p53 gene DNA is not a hot spot for AF mutagenesis as a 'late stage event' in human hepatocellular carcinogenesis. Other authors (*54*) discuss the bioactivation of AFs and sterigmatocystin by human liver cytochrome P_{450} enzymes to their genotoxic epoxides and the biological properties of AF-DNA adducts.

Routes of Exposure

Zarba *et al.* (*55*) studied DNA adduct formation in the liver of rats following either intratracheal injection or nasal aerosol inhalation exposure of AFB_1. Aerosol inhalation is an effective route of exposure to AFB_1 in rats and hamsters that results in genotoxic damage in the liver. Binding of intratracheal administered $[^3H]AFB_1$ to rat liver DNA is only marginally higher than that observed with hamster liver, in contrast to the wide difference observed in animals receiving AFB_1 intraperitoneally.

The lungs are the second most important organ after the liver to retain a considerable amount of [^{3}H]AFB_1 (66%). AFB_1 binding to hepatic DNA approached a peak at 1 h after intratracheal administration of the toxin to rats and hamsters (*56*). The persistence of AFB_1 binding to pulmonary DNA and the extent of translocated AFB_1 binding to hepatic DNA present an interesting difference from that observed when the toxin was administered through a gastrointestinal route. Alveolar macrophages possess specific mixed function oxidase activity to epoxidize AFB_1 (*56*). On the other hand, the route of administration of the carcinogen did not affect DNA binding over time in sprouts-fed animals (*17*).

Putt *et al.* (*57*) found that the nasal mucosa of some mammalian species are susceptible to the toxicity of AFB_1, and they studied the nasal enzymes involved in the metabolic activation of AFB_1 or the metabolites produced with nasal microsomes. Members of the P_{450} 2A gene subfamily play an important role in the metabolic activation of AFB_1 in rabbit and rat nasal mucosa and suggest a molecular basis for assessing the health risk associated with inhalation exposure to this procarcinogen in humans. Putt *et al.* (*57*) reported that the rates of AFB_1-N^7-guanine DNA adduct formation with rabbit and rat nasal microsomes (NMa) are over 3- and 10-fold higher, respectively, than with liver microsomes from the same species. On the other hand, the rates of formation of AFM_1 (9a-hydroxy-AFB_1) and AFQ_1 (3-hydroxy-AFB_1), products known to be less toxic, are lower with nasal than with liver microsomes. Nasal microsomes produce high levels of six unidentified polar metabolites that are not formed by microsomes from liver or several other tissues. Furthermore, the formation of AFB_1-DNA adducts by nasal microsomes is decreased by nicotine, a known inhibitor of P_{450} NMa (*57*).

Vitamins

The relationship of AFB_1-adducts and vitamins shows the association of urinary AFB_1-DNA adducts with the plasma levels of cholesterol, alpha-tocopherol (a form of vitamin E), lycopene (anti-oxidant red plant pigment), and alpha-carotene (one of the most abundant carotenoids; it is a "provitamin A" compound) and beta-carotene (a precursor of vitamin A) was observed at different exposure levels of AFB_1. No association with the adducts was found for plasma levels of retinol and testosterone. Alpha-tocopherol (vitamin E) and beta-carotene enhanced AFB_1-DNA adduct formation, and there is a relationship between plasma micronutrients and risk of AFB_1-related HCC (*34*). Opposite information reported that beta carotene was useful to control AFB_1 adduct formation (*58*).

Kinds of AF Adducts

Guanine Adducts

In vitro studies have shown that AFB_1 adducts form primarily at the N^7 position of guanine. The first report about structure of adducts was given by Essigman *et al.* (*59*) working with a combination of spectral and chemical data that indicated that the major product of the interaction of metabolically activated AFB_1 and DNA is 2,3-dihydro-2-(N^7-guanyl)-3-hydroxy-AFB_1 with the guanine and hydroxyl functions possessing a *trans* configuration. The structural data supported the hypothesis that the putative 2, 3-epoxide of AFB_1 was quantitatively important as an intermediate in the binding of AFB_1 to nucleic acids. These data were corroborated by Lin *et al,* (*60*) and Martin and Garner (*61*).

Essigmann *et al.*(*59*) found that the covalent binding of the hepatocarcinogen AFB_1 by rat liver microsomes to calf thymus DNA resulted in a binding level equal to one AF residue per 60 DNA nucleotides. An AF derivative-guanine adduct was efficiently liberated from DNA with formic acid. The major product of the interaction of metabolically activated AFB_1 and DNA, approximately 90% of the carcinogen bound to DNA, is AFB_1-N^7-Gua with hydroxyl functions possessing a *trans* configuration. The putative 8,9-epoxide of AFB_1 (AFB_1 oxide) is quantitatively important as an intermediate in the binding of AFB_1 to nucleic acids.

AFB_1, upon activation to a hypothesized AFB_1-2,3-epoxide reacts with DNA guanines. Aflatoxin B_1-2,3-dichloride (AFB_1-Cl_2) was originally synthesized as an electronic analog for AFB_1-epoxide, which has never been isolated due to presumed reactivity (*26*). Several types of adducts have been reported - a hemoglobin adduct formation by acrylonitrile in rats (*62*), benzo[a]pyrene DNA adducts generally considered a marker for the polycyclic aromatic hydrocarbons (PAH) content (*63*) and many others. In relation to AF-DNA adducts there are several kinds, and only the most frequent will be briefly described here.

Mapping the binding site of AFB_1 in DNA implies a systematic analysis of the reactivity of AFB_1 with guanines in different DNA sequences. The AFB_1 reacts almost exclusively at the N^7-position of guanine following activation to its reactive form, the AFB_1 epoxide. For AFB_1 epoxide: 1) the N^7-guanine adduct is the major adduct found in all of the DNA polymers; 2) adduct levels vary in different sequences, and thus sequence specificity is also observed; and 3) the intensity of bands in DNA sequencing gels is likely to reflect adduct levels formed at the N^7-position of guanine (*64*).

The only AF-DNA adducts detected in significant amounts are guanine adducts, irrespective of the type of AF used (AFB_1 or AFB_2) or the mode of its activation. No stable adduct with adenine, cytosine or thymine was detected. The major AFB_1-DNA photoadduct is again the N^7-guanine adduct (*65*).

Studies on the reaction of synthesized AFB_1-8,9-epoxide (*66*) with DNA *in vitro* strongly suggests that adduction *in vivo* proceeds via a pre-covalent intercalation complex between double stranded DNA and the highly electrophilic, unstable AFB_1-*exo*-8,9-epoxide isomer (*67*). The *in vivo* metabolism and hepatic adduction of AFB_1 in zebrafish showed that the adult fish can rapidly metabolize and excrete AFB_1 after intraperitoneal administration. This species can bioactivate AFB_1 and the resulting DNA adducts suggest sensitivity to this carcinogen (*30, 31*).

The guanine adducts are generally found as urinary AFB_1-DNA adducts and are useful as indicators of recent exposures (*68*). For such assays to be of more significant use, full validation studies are required in which the relationships between exposure dose, DNA dose, and excreted dose are elucidated. Studies to establish the relationship between exposures of rats to AFB_1 and their urinary excretion of AFB_1-N^7-guanine have been reported (*69*).

The detection and measurement of excretion rates of DNA adducts in urine may provide information on the subject's recent exposure and possibly give an indication of DNA repair in the individual. Therefore, studies of DNA adducts in urine may provide data complementary to those obtained from measurement of adduct levels in cellular DNA in the same individual. A problem in quantifying studies is that the dose of AF excreted in urine may not be wholly indicative of the fate of the retained internal dose.

Other minor N^7-guanyl adducts can arise through enzymatic oxidation of AFP_1, AFM_1 and other AFB_1 phase I metabolites unsaturated in the 8,9 position (*70*). According to the balance of specific P_{450} isozymes the production of such adducts would vary among species. Not all AFs with intact 8,9 double bonds form adducts directly. AFL is a highly carcinogenic phase I metabolite which acts as a reservoir for extending AFB_1 carcinogenicity in certain species, especially trout (*71*). Metabolic studies with AFL tritiated at the cyclopentenol carbon indicated at least 95% back-conversion to AFB_1 prior to DNA adduction (*72*). Subsequent studies have failed to identify any AFL-derived adduct other than 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy-AFB_1. Other authors (*23*) concluded that more than 99% of AFL-DNA adducts produced *in vivo* were identical to those produced by AFB_1. Thus similar molecular dosimetry responses should be expected under all exposure protocols in which the two parent carcinogens do not exhibit differing toxicities to the target organ (*23*). It is possible that the cyclopentol hydroxyl renders AFL a poor P_{450} substrate for oxidation and that the 8,9 epoxide, if formed, is unable to form an effective pre-covalent intercalation intermediate with DNA.

The primary DNA adduct is an N^7-guanine adduct. The kinetics of the loss of this guanine and its transformation into the more stable secondary adduct resembled that reported recently for the major primary DNA adduct formed by the reaction of AFB_1 at the N^7-position of guanine in the DNA of normal cells and its transformation into the putative AFB_1-ring opened triamino pyrimidyl structure (*73*).

FAPY Adducts

Another adduct that is related to the presence of a positive charge on the imidazole portion of the initial N^7-guanyl adduct gives rise to a ring-opened formamidopyrimidine (FAPY) derivative with distinct chromatographic behavior (*74*) and the metabolic activation of other AF metabolites, such as AFM_1 and AFP_1. Subsequent conversion of these initial N^7-guanyl adducts to FAPY derivatives can add complexity to AF-DNA adduct chromatographic profiles (*70*).

Accumulation of the FAPY derivative is time dependent and non-enzymatic. This adduct is more important in the studies of cancer - although is it less frequent in the DNA, it persists for a longer time and this gives it more potential biological importance because of its apparent persistence in DNA.

The presence of the FAPY AF-DNA adduct, was investigated *in vivo* by immunohistochemical analysis in 14 paired hepatocellular carcinoma and non-tumorous human liver tissue sections using a monoclonal anti-AFB_1-formamidopyrimidine antibody (*33*). The highest level of covalent products was present approximately 24 h after exposure to 0.5 ppm AFB_1. Ninety-six hours later, this level had decreased slightly; however, the distribution of covalent adducts had changed and FAPY adducts now represented up to 50% of the hydrolyzed AF derivatives.

The metabolism and formation of AF-DNA adducts in rainbow trout (*Oncorhynchus mykiss*) and their use as molecular dosimeters for tumour prediction have been reported (*75*). The comparative studies with structurally related AFs, with *Coho salmon* and rodents using the "molecular dosimetry" concept, have also been discussed (*75*).

Protein Adducts

Hemoglobin Adducts

Protein adducts in blood can be used for estimating exposures to electrophilic carcinogens. Hemoglobin has been used due to its abundance and the long life, 120 days, of human erythrocytes. Hemoglobin adducts are stable and can be detected many weeks after exposure, thus facilitating retrospective monitoring. Where chronic exposures occur, the amino acid adducts in hemoglobin accumulate and a measure of the integrated dose of the erythrocyte can be determined (*76, 77*).

Albumin Adducts

Albumin is another abundant blood protein which has also been recognized as a potentially useful dose monitor. An advantage of albumin is that it is synthesized in the hepatocyte, a site where many carcinogens are metabolized to their reactive forms. Albumin is a component of the interstitial fluid surrounding all cells that can react with electrophilic metabolites from any tissue. Adduct levels formed in serum albumin exhibit similar dose dependence as hemoglobin. However, albumin in man has a shorter lifetime, 20 days, than hemoglobin. In the case of food carcinogens, the binding of AFB_1 to albumin is greater than to hemoglobin; therefore serum albumin is a more effective monitor than hemoglobin for food related carcinogens.

The intrinsic reactivity of the protein may show differences in different species thus making interspecies extrapolations difficult. Protein adducts are relatively long-lived and are not repaired. The formation of adducts in the protein can give rise to unstable adducts and may destabilize the protein.

The detection of protein adducts indicates exposure and these adducts are valuable in the identification of hazards. In the case of low dose exposures, which are relevant to man, there is a proportionality in the formation of protein and DNA adducts. In cases where the relationship between protein alkylation at the critical target site has been rigorously established then protein adducts can be used in risk estimation.

Lysine Adducts

AFB_1-albumin data indicate a mean daily exposure of AF in blood, and AFB_1-lysine is a digestion product of AFB_1-albumin which has been used in animal and human studies (*78*). This adduct has been important in developing exposure and internal adduct relationships in humans.

Measurable internal AFB_1 exposure may be occurring in some United Kingdom individuals, although at lower levels than those seen from areas with high AFB_1 exposure. For United Kingdom human sera, the mean adduct levels were 29.3 +/- 14.8 pg AFB_1-lys equivalents (eq)/mg albumin (males) and 26.9 +/- 14.4 pg AFB_1-lys eq/mg alb (females). The source of this exposure may reflect difficulties in accurately monitoring regulated imported foodstuffs or the lack of regulations of other contaminated imports. The mean daily exposure in the United Kingdom is 3 μg of AFB_1 with a mean internal dose in liver DNA of 5.9 adducts/10^7 nucleotides (*78*).

To facilitate the study of AFB-lysine adducts, mouse monoclonal antibodies were developed against a synthetic AFB_1-lysine-cationized bovine serum albumin conjugate (*79*). The isotype of one of these antibodies, IIA4B3, has been classified as immunoglobulin G_1 (lambda). The affinities of IIA4B3 for AFB and its associated adducts and metabolites are ranked as follows: AFB-lysine > 8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydro pyrimid-5-yl formamido)-9-hydroxy-AFB > AFB = 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy-AFB > AFM_1 > AFQ_1. IIA4B3 had about a 10-fold higher affinity for binding to AFB-lysine adduct than to AFB when [^{3}H]AFB-lysine was used as the tracer. The concentration for 50% inhibition for AFB-lysine was 0.610 pmol; that for AFB was 6.85 pmol. IIA4B3 had affinities at least sevenfold and twofold higher than those of 2B11, a previously developed antibody against parent AFB, for the major AF-DNA adducts 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy-AFB and 8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl-formamido)-9-hydroxy-AFB, respectively. An analytical method based on a competitive radioimmunoassay with IIA4B3 and [^{3}H]AFB-lysine was validated with a limit of detection of 10 fmol of AFB-lysine adduct. The method has been applied to the measurement of AFB-albumin adduct levels in human serum samples collected from the residents of areas at high risk for liver cancer (*79*).

In relation to the adduct levels as product of the addition of several doses there is a study (*25*) describing an experiment with male F-344 rats dosed with [^{3}H]AFB$_1$ in the drinking water at 3 exposure levels (0.02, 0.6, 20 μg/L, resulting in mean dose levels of 2.2, 73, 2110 ng/kg daily). After 4, 6, and 8 weeks, DNA was isolated from the livers and analysed for AF-DNA adducts. The level of DNA adducts did not increase significantly after 4 weeks, indicating that a steady-state for adduct formation and removal had nearly been reached. At the high dose level (2,110 ng/kg daily), a near 50% tumor incidence would be expected in a 2-year bioassay with F-344 rats while the low dose (2.2 ng/kg daily) used is within the range of estimated human dietary exposures to AF in Western countries. The proportionality seen between exposure and steady-state DNA adduct level is discussed with respect to a linear extrapolation of the tumor risk to low dose (*25*).

Wild *et al.*(*80*) made studies to (a) correlate AFB$_1$ serum albumin adduct levels with AFB$_1$-DNA adduct levels in liver in different rodent species (the sensitive rat and the resistant hamster and mouse) to determine whether the former could serve as a marker of hepatic DNA adduct levels irrespective of the species, and (b) to relate the levels of both adducts to differences in susceptibility to tumor induction by AFB$_1$ in the different species. Finally they compared the dose response for AFB$_1$-albumin adduct formation in the rodent species with that in human populations exposed environmentally to AFB$_1$.

The levels of AFB$_1$-albumin adduct also reflect at least qualitatively the relative susceptibility of the different species to AFB$_1$ carcinogenesis. Data from The Gambia and southern China (*80*) suggest that humans exposed to AFB$_1$ form amounts of albumin adducts, and by extrapolation amounts of DNA adducts, closer to those observed in AFB$_1$-sensitive rat species (0.3 and 0.51 pg AFB$_1$-lysine equivalent/mg albumin/μg AFB$_1$/kg body weight) and 1-2 orders of magnitude higher levels (1.56 pg/mg albumin) than hamster and mouse (<0.025 pg AFB$_1$-lysine), the AFB$_1$-resistant species.

Anwar *et al.*(*81*) studied the micronuclei, chromosomal aberrations, and AF-albumin adducts in experimental animals after exposure to AFB$_1$. Rats and mice differ markedly in sensitivity to AFB$_1$ hepatocarcinogenicity, the former being sensitive and the latter resistant. Both chromosomal aberrations and micronuclei were significantly increased in treated rats compared to the control group at doses above 0.1 μg/g. Mice presented a slight increase in chromosome aberrations in the highest dose group (1.0 μg/g) but no increase in micronuclei was observed at any of the doses. The AF-albumin adduct may reflect the level of genetic alteration resulting from the initial binding of this carcinogen to cellular DNA. Therefore, using this adduct as a biomarker in studies of human exposure to AF may provide information not only on exposure but also on the risk of genetic alterations consequent to that exposure.

Adenosine and Cytosine AFB_1 Adducts

Minor adduction of AFB_1 to adenosine (*82*) and cytosine (*83*) in DNA *in vitro* has been reported, but evidence is lacking that these adducts form *in vivo* or have importance in AF carcinogenesis. Other biomarkers are lymphocyte DNA and serum albumin adducts.

Methodology

There are different methods to study AF-DNA adducts, although the first step is sampling. In the case of the extraction of AFs from samples of different plant/animal origin, they have to be well homogenized to be representative of the total lot. In the case of foods, feeds, grains, or other plant derivatives, a subsample of 50 g has to be blended with organic solvents (methanol, chloroform, or acetonitrile) to obtain a good extraction, then filtered. The concentration and purification of the filtrate is obtained with immunoaffinity columns that have the anti-aflatoxin antibody mixed with agarose. The filtrate generally is diluted with phosphate buffer saline (PBS) at pH 7.4, so that the antibody is pH protected and it is recommended that the column receives 1 g of sample.

Immunoaffinity columns have to be activated by previously passing 20 mL of PBS at pH 7.4, because the specific AF antibodies contained in the agarose are very sensitive to other acidities. Next the column receives the AF from the diluted sample filtrate retaining the AF by an antigen-antibody reaction and the column is washed with distilled water. Finally, AF are eluted from the immunoaffinity column with 2 mL of pure methanol, but also acetonitrile or chloroform can be used.

In the case of AF-DNA adducts, first the sample (urine, blood, animal or human tissues) has to be homogenized, then the DNA is purified and digested with protease A, getting the RNA away with RNA nucleases. To cut the DNA, it is hydrolyzed with boiling HCl, until the DNA is depurinated. Finally an aflatoxin competitive inhibition ELISA method is used (*84*). This kind of ELISA detects traces of AF-adducts (picograms or femtograms).

The most common methods to analyze AF-adducts are brieftly described in the following paragraphs.

Reversed-phase High-pressure Liquid Chromatography (LC)

LC is the main **procedure** to identify and quantify AF adducts. Essigmann *et al.* (*59*) studied the covalent binding of the AFB_1 by rat liver microsomes to calf thymus DNA and found a binding level equal to one AF residue per 60 DNA nucleotides. An AF derivative-guanine adduct was efficiently liberated from DNA with formic acid. Analytical LC of the DNA hydrolysate revealed that approximately 90% of the carcinogen bound to DNA could be accounted for as a single component. A combination of spectral and chemical data indicates that the major product of the interaction of metabolically activated AFB_1 and DNA is AFB_1-N^7-Gua with hydroxyl functions possessing a *trans* configuration. The structural data support the hypothesis that the putative 2,3-epoxide of AFB_1 is quantitatively important as an intermediate in the binding of AFB_1 to nucleic acids.

Sotomayor *et al.* (*85*) studied dose-response and temporal patterns of DNA and RNA adduct formation in rat liver due to intermittent exposure to AFB_1; AFB_1-DNA and -RNA adducts were measured by LC with fluorescence detection. The ratio of AFB_1 to hepatic DNA was a linear function of the dose, regardless of the way this was administered. The dose-response relationship for RNA adducts depends on the length of the no-dosing cycles and on the turnover rate of RNA.

Liquid Chromatography Electrospray Tandem Mass Spectrometry (LC-ESI-MS/MS)

LC-ESI-MS/MS is a procedure for the measurement of aflatoxin biomarkers in urine which has been developed and validated (*86*). The two major aflatoxin-DNA adducts formed in rat tissues, aflatoxin N^7-guanine and its imidazole ring opened derivative AFB_1-FAPY, were detected and quantified in urine by the LC-ESI-MS/MS technique. This has important implications for the application and analysis of the efficacy of primary and secondary prevention interventions in human populations where ambient exposure levels are low, but the toxicological hazards of these exposures remain high.

Polymerase Chain Reaction

The methods utilizing quantitative PCR analysis (QPCR) and ligation mediated polymerase chain reaction (LMPCR) provide means to assess gene-specific and sequence-specific AFB_1 damage. The method and analysis also prove that microsomally-mediated damage is a suitable method for avoiding manipulations with very unstable DNA-reactive metabolites and that this damage can be detected by QPCR and LMPCR.

The affinity and specificity of the isotope of the antibody IIA4B3 was further characterized by a competitive radioimmunoassay (*78*).

Radioactive Methods

Radioactive methods are also used and some examples of their use are presented here. Shaulsky *et al.* (*65*) found that AF-DNA adducts were formed by microsomal and photoactivation systems, using nick-translated DNA labelled with [^{14}C] in each of the DNA bases and reaction with [^{3}H]AFB$_1$ and [^{3}H]AFB$_2$. DNA adducts were analyzed by LC of DNA hydrolysates and were characterized as double labelled peaks with specific retention times. The only AF-DNA adducts which were detected in significant amounts were guanine adducts, irrespective of the type of AF used or the mode of its activation. No stable adduct with adenine, cytosine, or thymine was detected. UV spectra, proton nuclear magnetic resonance (NMR) spectroscopy and mass spectrometry (MS) were consistent with the notion that the major AFB$_1$-DNA photoadduct is the N^7-guanine adduct.

Electron Spin Resonance (ESR) Spectroscopy

Towner *et al.* (*38*) identified the trapped free radical intermediates from the *in vivo* hepatic metabolism of AFB$_1$ in rat bile via electron spin resonance (ESR) spectroscopy *in vivo* following AFB$_1$ metabolism using the spin trapping α-(4-pyridyl 1-oxide)-*N*-*tert*-butylnitrone (4-POBN) technique.

Enzyme-linked Immunosorbent Assay (ELISA) with Monoclonal Antibodies

ELISA methodology is possible thanks to the technology for making monoclonal antibodies. High-affinity monoclonal antibodies for AF and their application to solid-phase immunoassays and also the production of a high affinity IgM monoclonal antibody that recognizes AFB$_1$, AFB$_2$, AFM$_1$ and major AF-DNA adducts have been reported. The antibody was used to construct a reusable antibody affinity chromatographic column capable of isolating aflatoxins from serum, urine, and milk (*87*).

Monoclonal antibodies and dietary antioxidant based animal models have been applied to define human exposure to aflatoxin B$_1$. The use of monoclonal antibodies for quantifying aflatoxins, particularly the aflatoxin adduct AFB-N^7-Gua in biological fluids, particulary urine, and their applicability to biochemical epidemiological studies in humans have been discussed, together with the use of animal models based on the differential effects of ethoxyquin on the kinetics of AF-DNA adduct and GGT-positive foci formation to assign risk to people. It is concluded that measurement of the major, rapidly excised adduct is an appropriate dosimeter for estimating exposure status and risk in individuals consuming this mycotoxin (*88*).

Groopman and Kensler (*89*) explained the use of monoclonal antibody affinity columns for assessing DNA damage and repair following exposure to AFB_1. Monoclonal antibodies specific for AF metabolites, especially DNA adducts, were developed and used in a reusable monoclonal antibody affinity chromatography column for the rapid isolation of metabolites from biological fluids. Urine samples from people exposed to AFB_1 dietary sources were analysed. Measurement of the major, rapidly excised, B_1-N^7-Gua adduct in tissues and fluids is an appropriate means for estimating exposure status and risk in individuals consuming aflatoxins.

Pestka *et al.* (*90*) worked on the reactivity of AFB_{2a} antibody with AFB_1-modified DNA and related metabolites. Aflatoxin B_{2a} (AFB_{2a}) antiserum has been previously used in an ELISA for the quantitation of AFB_1 and AFB_{2a}. The results showed that estimates of the kinetics and substrate dependence of covalent binding to calf thymus DNA in rat microsomal incubation mixtures by both methods were comparable. The broad specificity AFB_{2a} antibody might be of considerable value in the detection of AFB_1 macromolecular adducts and related metabolites in epidemiological investigations or in the diagnosis of aflatoxicosis.

Garner *et al.* (*91*) used immunoassay procedures to detect exposure to AFB_1 and benzo[a]pyrene in animals and man at the DNA level.

Hsieh *et al.* (*9*) developed monoclonal antibodies after fusion of mouse myeloma cells (Sp_2) with spleen cells and developed an immunological detection of AFB_1-DNA adducts formed *in vivo*. Two monoclonal antibodies (6A10 and 12F5) were obtained after fusion of mouse P3X63-AG.8.653 myeloma cells with spleen cells isolated from BALB/c mice immunized with imidazole ring-opened AFB_1-DNA and characterized by competitive enzyme-linked immunosorbent assays. In a pilot study, AFB_1 adducts were detected in liver tissues from individuals living in areas with suspected exposure to AFB_1. Monitoring adduct levels in human tissue may provide information not only on carcinogen exposure but also on the relationship among infection with hepatitis B virus, dietary exposure to AFB_1,and liver cancer (*9*).

Chu (*92-95*) developed methods for preparation of AF-protein conjugates and production and characterization of antibodies against aflatoxins. He reviewed and compared the use of AF antibodies in methods of analysis of foods and feeds for AF content, including radioimmunoassay, competitive, direct and indirect competitive ELISAs, rapid immunoscreening tests, immunoaffinity chromatography, and immunochromatography. Immunochemical methods of monitoring human exposure by measuring AF metabolites in urine and milk, AF-DNA adducts in urine, tissues, AF in serum, and immunohistochemical methods were all implemented.

The use of monoclonal antibody affinity columns is necessary for assessing DNA damage and repair following exposure to AFB_1. Further studies employing different classes of modifiers of aflatoxin carcinogenesis in rodent models should better define the relationships between hepatic and urinary levels of AFB_1-N^7-Gua and susceptibility to neoplasia (*89*).

The presence of AFB_1-FAPY, a persistent AF-DNA adduct, was investigated *in vivo* by immunohistochemical analysis in 14 paired hepatocellular carcinoma and nontumorous human liver tissue sections using a monoclonal anti-AFB_1-FAPY antibody (*33*). *In vitro* adduct formation and cellular DNA was investigated with a modified DNA immunoblot assay (*33*).

A simple, rapid, and highly sensitive indirect competitive enzyme-linked immunosorbent assay (ELISA) was reported to determine AFB_1-DNA adducts and urinary AFB_1-N^7-guanine adduct. The proposed method may find application in the biological monitoring of AFB_1 in molecular epidemiological studies to assess the dietary human exposure of AFs (*96, 97*).

When Santella *et al.* (*98*) determined the role of exposure to AF in the induction of hepatocellular carcinoma (HCC) in Taiwan, they employed immunohistochemical detection of AFB_1-DNA adducts and hepatitis B virus antigens in HCC and non-tumorous liver tissue. They found that there was no association between AFB_1-DNA adducts and HBsAg and HBxAg.

Finally, another kind of methodology called immunoslot blotting was useful for adduct quantitation, in order to analyze dose-response relationships for hepatic AFB_1-DNA adduct formation in the rat *in vivo* and *in vitro* (*99*).

Indirect Immunofluorescence Analysis

The quantitation of AFB_1-DNA adducts in woodchuck hepatocytes and rat liver tissue was done by indirect immunofluorescence analysis (*100*). A quantitative indirect immunofluorescence technique was developed utilizing a monoclonal antibody (6A10) recognizing the imidazole ring-opened form of the major N^7-guanine adduct of AFB_1. This method was used to investigate adduct formation in woodchuck hepatocytes treated in culture and in liver tissue of rats treated i.p. with AFB_1. Adduct levels were quantitated by competitive ELISA with antibody 6A10 and by fluorescence spectroscopy. There was a significant correlation of the quantitative immunofluorescence intensity with levels of AFB_1 adducts detected by ELISA ($r = 0.61$, $P < 0.05$) and spectrofluorescence ($r = 0.78$, $P < 0.01$). This immunohistochemical method should be applicable to the detection of adducts in liver tissues of humans exposed to high levels of dietary AFB_1 (*100*).

Ames Test

Choi *et al.* (*36*) reported a comparative assessment of DNA adduct formation, Ames test *Salmonella* mutagenicity test, and chromosome aberration assays using AFB$_1$ as short-term tests for DNA damage. DNA adduct formation assay *in vitro* could be useful for detecting genotoxic compounds (*36*).

The Ames test was also used in studies on the inhibition of AFB$_1$ mutagenesis in *Salmonella typhimurium* and DNA damage in cultured rat and human tracheobronchial tissues by ellagic acid (*101*).

Effects

Aflatoxicoses cause a variety of disease manifestations which are related to the ability of AFs to impair protein synthesis and react with the macromolecules and cellular organelles and to interfere with the normal production of cellular regulators. Severe AF poisoning causes hepatic necrosis, coagulation defects, vomiting, severe hemorrhages, and death. Other chronic effects in the human being are immunosuppression, thymic aplasia, hepatitis, cirrhosis, cancer, and Reye's syndrome (*102*). AFs are the most toxic mycotoxins acting in traces and they damage all animals (cattle, poultry, horse, fish, rats, dogs, cats, etc.). They also contaminate plants (cereals, spices, oilseeds, figs, dry fruits, etc.) and their derived products (eggs, milk, dairy products such as cheese and cream, also wine, beer, feeds, foods from cereal, and many others (*102*).

Epidemiological evidence indicates that AFB$_1$ intake is associated with an increased risk of hepatocellular carcinoma (HCC) (*34*).

Low levels of AFB$_1$ metabolism by intact mouse and hamster mitochondria and the relative resistance of macromolecular synthesis in these particles to added AFB$_1$ may be due to mitochondrial membrane impermeability. In support of this possibility, AFB$_1$ transported into mouse liver mitochondria through a liposome delivery system caused about 80% inhibition of protein synthesis (*103*).

Barraud *et al.* (*104*) used the Pekin duck model to investigate the interactions between hepadnaviral infection and AFB$_1$ exposure in the induction of oxidative stress in the liver. AFB$_1$ exposure of Pekin ducks infected with duck hepatitis B virus induced a significant increase in viral replication associated with an intense biliary ductular cells proliferation. Extremely high levels of AFB$_1$-DNA adducts (40-120 pmol AFB$_1$-FAPY/mg DNA) and AFB$_1$-albumin adducts (1,500-3,000 pg AFB$_1$-lys eq/mg albumin) were detected in duck liver and serum, respectively, as compared to other animal species exposed to a similar AFB$_1$ dose. Duck hepatitis B virus infection was found to induce a non-significant increase in AFB$_1$-albumin adduct levels in duck serum. In terms of hepatic antioxidant enzymes (catalase, superoxide dismutase,glutathione peroxidase) a significant increase in superoxide dismutase activity occurred following AFB$_1$ exposure, but not of duck hepatitis B virus infection, but this was observed only after the cessation of treatment, when biliary ductular cells proliferation was reduced (*104*).

Control

There are several options to diminish or control the problem of the presence of adducts in the DNA of an organism that can cause a mutation and maybe a carcinogenic process. These possibilities go from natural repair rates, implicated enzymes, chemicals, and natural products.

Natural Repair Rates and Adduct Removal

Natural repair rates in the hamster and rat were constant over time with removal of the 8,9-dihydro-8-(N^7-guanyl)-9-hydroxy AFB$_1$ accounting for the majority of adduct disappearance. The rabbit demonstrated biphasic repair of adducts; 8,9-dihydro-8-(2-amino-6-formamido-4-oxo-3,4-dihydropyrimid-5-yl-amino)-9-hydroxyl-AFB$_1$ was rapidly removed during the first 12 h post-treatment with AFB$_1$, followed by a slower removal phase of primarily 8,9-dihydro-8-N^7-guanyl-9-hydroxy-AFB$_1$. Carcinogen activation and repair capabilities of tracheal epithelium vary among species (rabbit > hamster > rat), and these processes likely relate to the presence of smooth endoplasmic reticulum containing non-ciliated tracheal epithelial cells in those species (*33*).

Induction of resistance to AFB$_1$ binding to cellular macromolecules in the rat by chronic exposure to AFB$_1$ and AFM$_1$ was investigated. Pre-exposure to AFM$_1$ resulted in a small reduction in binding to nucleic acids (*105*).

Genotoxins in mixture provoke DNA-damaging effects. There was a modulation of DNA adduct formation by pre-existing adducts. AFB$_1$-binding to DNA may be altered by conformational changes in the helix, due to the presence of a pre-existing acetylamino-fluorene adduct. There is absence of an effect by AF and confirmation of local denaturation of the oligomer helix by use of the chemical probes hydroxylamine and diethylpyrocarbonate. Changes in the nucleophilicity of neighboring nucleotides and local steric effects cannot be ruled out (*27*).

Detoxification Enzymes

Glutathione transferase activity was increased by 133% in animals fed 50 ppb AFB$_1$, by 48% in those preexposed to 50 ppb AFM$_1$, and remained at control values in rats fed 0.5 ppb AFM$_1$. These results suggest that the induction of detoxification enzymes following chronic exposure to aflatoxin might contribute to the reduction in covalent binding of AFB$_1$ to macromolecules (*105*).

Some definitions are pertinent in order to understand the full meaning of enzyme inhibition: 1) aryl hydrocarbon (Ah) receptor is a cytosolic protein which is an activator of transcription that increases the abundance of selective cytochrome P$_{450}$s; and 2) agonist is a substance that binds to a specific receptor and triggers a response in the cell. It mimics the action of an endogenous ligand (such as hormone or neurotransmitter) that binds to the same receptor (*106*). In the rainbow trout, enzyme inhibition by beta-naphthoflavone (BNF) is an Ah receptor agonist against AFB_1 8,9-epoxidation and is the sole protective mechanism at low doses and the predominant protective mechanism at higher BNF doses. Diets with BNF inhibited *in vivo* AFB_1-DNA adduction by 46%. Mechanisms of chemoprevention can change with anticarcinogen dose, and caution that even potent induction of phase I or phase II activities does not assure that pathway is a predominant protective mechanism *in vivo* (*107,108*).

Im *et al.* (*109*) reported on the modulation of AFB_1 biotransformation by BNF in isolated rabbit lung cells. The cytotoxic and carcinogenic mycotoxin AFB_1 is biotransformed by the cytochrome P_{450} monooxygenases (CYP) to a number of relatively nontoxic metabolites, as well as to the ultimate toxic metabolite, AFB_1-8,9-epoxide. In a number of tissues and species, AFB_1 hydroxylation to the relatively non-toxic metabolite, AFM_1, is induced by BNF treatment. Although the liver is the principal target organ for AFB_1 toxicity, the mycotoxin is also toxic and carcinogenic to respiratory tissues. The decrease in AFB_1-DNA binding from rabbits treated with BNF is apparently due to the selective induction of CYP isozymes and related increases in AFM_1 formation, and not to direct inhibition of epoxidation or enhanced conjugation of AFB_1-8,9-epoxide with glutathione (*109*).

Among members of the mouse cytochrome P_{450} 2A family, P_{450} 2A5 is the best catalyst of AFB_1 oxidation to its 8,9-epoxide (*110*). There are studies about the role of amino acid residues 209 and 365 of the P_{450} 2A5 in the metabolism and toxicity of AFB_1 using recombinant yeasts. In addition, replacing the hydrophobic amino acid at the 365 position with a positively charged lysine residue strongly decreased the metabolism of AFB_1. The changes in AFB_1 metabolism contrast with the changes in coumarin 7-hydroxylation caused by these amino acid substitutions, since reducing the size of the 209 residue strongly reduced coumarin metabolism and increased the K(M) values. The catalytic parameters of AFB_1 correlated generally with its toxicity to the recombinant yeasts expressing the activating enzyme and with the binding of AFB_1 to yeast DNA. Furthermore high affinity substrates and inhibitors (e.g., methoxsalen, metyrapone, coumarin 311, 7-methylcoumarin, coumarin, and pilocarpine) of P_{450} 2A5 could efficiently block the toxicity of AFB_1. The recombinant yeasts expressing engineered P_{450} enzymes are a useful model to understand the substrate protein interactions, to study the relationship of metabolic parameters to toxicity, and to test potential inhibitors of metabolism based toxicity (*111*).

Induction of resistance to AFB_1 binding to cellular macromolecules in the rat by chronic exposure to AFB_1 and AFM_1 was investigated (*105*). Pre-exposure to AFM_1 resulted in a small reduction in binding to nucleic acids. In rats pre-exposed to 50 ppb AFB_1, glutathione transferase activity was increased by 133%, and binding of labeled AFB_1 to DNA, RNA, and protein was decreased by 72%, 74%, and 61%, respectively. In rats pre-exposed to 50 ppb AFM_1, binding decreased 48% and remained at control values in rats fed 0.5 ppb AFM_1. The induction of detoxification enzymes following chronic exposure to AF might contribute to the reduction in covalent binding of AFB_1 to macromolecules (*105*).

The AFB_1 aldehyde metabolite may contribute to the cytotoxicity of AFB_1 via protein adduction. AFB_1 aldehyde reductases, specifically the NADPH-dependent aldo-keto reductases of rat (AKR7A1) and human (AKR7A2), are known to metabolize the AFB_1 dihydrodiol by forming AFB_1 dialcohol. Using a rat AKR7A1 cDNA, Knight *et al.* (*112)* isolated and characterized a distinct aldo-keto reductase (AKR7A3) from an adult human liver cDNA library. The deduced amino acid sequence of AKR7A3 shares 80 and 88% identity with rat AKR7A1 and human AKR7A2, respectively. There are varying levels of expression of AKR7A RNA in human liver and in several extrahepatic tissues, with relatively high levels in the stomach, pancreas, kidney, and liver. Based on the kinetic parameters determined using recombinant human AKR7A3 and AFB_1 dihydrodiol at pH 7.4, the catalytic efficiency of this reaction (k2/K, per M/s) equals or exceeds those reported for other enzymes, for example cytochrome P_{450}s and GST, known to metabolize AFB_1 *in vivo*. Depending on the extent of AFB_1 dihydrodiol formation, AKR7A may contribute to the protection against AFB_1-induced hepatotoxicity (*112*).

Inhibition of AFB_1 Lesions by Different Compounds

AFB_1-induced tumors or preneoplastic lesions in experimental animals can be inhibited by co-treatment with several compounds, some of which are described here.

Fischer 344 rats readily develop liver cancer when exposed to AFB_1 but dietary administration of the antioxidant ethoxyquin (EQ) provides protection against hepatocarcinogenesis (*113*). Chemoprotection by EQ is accompanied by the overexpression of enzymes which detoxify activated AFB_1. AF-protein adduct formation takes place following metabolism of AFB_1 to the dialdehydic form of AFB_1-dihydrodiol. The dialdehyde can be detoxified by reduction to a dialcohol through the catalytic actions of an enzyme present in the hepatic cytosol from rats fed EQ-containing diets; this metabolite is undetectable in reaction mixtures that use hepatic cytosol from rats fed control diets. The enzyme responsible for catalyzing the formation of dihydroxy-AFB_1 has been purified from the livers of rats fed on diets supplemented with EQ. It is a soluble monomeric protein and this inducible enzyme has been designated AFB_1-aldehyde reductase (AFB_1-AR), a previously unrecognized enzyme that could provide protection against the cytotoxic effects of AFB_1 resulting from the formation of protein adducts. The relative importance of AFB_1-AR and the glutathione-S-transferase Yc2 subunit in conferring resistance to AFB_1 is discussed by Hayes *et al.* (*113)*.

Chemoprevention and Protective Chemical Compounds

Cancer chemoprevention is the use of agents to inhibit, delay, or reverse carcinogenesis. A number of potential targets for chemoprevention have recently been identified. Many classes of agents including anti-estrogens, anti-inflammatories, antioxidants and other diet derived agents have shown a great deal of promise (*114*).

Dietary Dithiolethione (DTT)

This substance proved to be a potent protector against AFB_1 hepatocarcinogenesis. It also reduced levels of hepatic AFB_1 (AFB)-DNA adducts following acute or subchronic (days 0-4, 7-11) treatments with AFB (250 µg/kg daily) by 80%, because it increased the hepatic activity of the Phase II enzyme GST without any effect on cytochrome P_{450} levels or on Phase I enzyme activities. Although total urinary excretion of AFB equivalents was unchanged in control vs DTT rats, the elimination of the major DNA adduct, AFB-N^7-guanine, was markedly reduced in animals fed DTT (*115*).

Butylated Hydroxytoluene (BHT)

BHT is known (*116*) to inhibit tumor formation due to AFB_1, by induction of liver GSH-S-transferases. High doses of BHT (0.75% for 15 days in diet) is associated with a pathological effects, but the permitted dose of BHT, added to processed food as a preservative, plays no role in the biotransformation of AFB_1. Allameh (*116*) compared the effect of low- and high-dose dietary BHT on microsome-mediated AFB_1-DNA binding.

To elucidate biochemical mechanisms underlying the anticarcinogenic activity of BHT, studies were undertaken to characterize the influence of BHT pretreatment on the metabolism and genotoxicity of AFB_1 in primary cultures of rat hepatocytes (*117*). The anticarcinogenic activity of BHT is due in part to preferential enhancement of hepatic detoxification mechanisms, with the result that intracellular concentrations of reactive metabolites are reduced and fewer covalently bound adducts are formed (*117*).

Indomethacin produced a 63-100% decrease in $[^3H]AFB_1$-DNA binding in macrophages from five of seven patients, while nordihydroguaiaretic acid inhibited the $[^3H]AFB_1$-DNA adduct formation by 19, 40, and 56% in macrophages from three of seven patients (*16*).

Selenium could effectively inhibit AFB_1-induced DNA damage, which may be partially responsible for its anticarcinogenic effect against AFB_1. Selenium pretreatment resulted in a dose-dependent inhibition of AFB_1-DNA binding as well as adduct formation. This was accompanied by an increase of reduced GSH in the liver of selenium-treated animals (*118*).

Natural Nutrients

Brussels sprouts significantly ($P < 0.001$) decreased hepatic AFB_1-DNA binding by 50-60% and increased hepatic and intestinal GST activities (*17*).

Oltipraz [5-(2-pyrazinyl)-4-methyl-1,2-dithiole-3-thione; RP 35972] is a synthetic, substituted 1,2-dithiole-3-thione previously used in humans as an antischistosomal agent. Cruciferous vegetables (e.g., Brussels sprouts, cabbage) contain several agents, including dithiolethiones, which appear to inhibit carcinogenesis; however, it is unclear which dietary compounds produce the protective effects (*119*). Animal studies have demonstrated that Oltipraz is a potent inducer of Phase II detoxification enzymes, most notably GST. Dietary concentrations of Oltipraz produce marked inhibition of AFB_1-induced hepatic tumorigenesis in rats. Levels of hepatic AF-DNA adducts, urinary AF-N^7-guanine, and serum AF-albumin adducts decreased when biliary elimination of AF-glutathione conjugants increased, thus providing predictive biomarkers that measured a chemopreventive effect. In other animal experiments, Oltipraz was found to inhibit chemically induced carcinogenesis in bladder, colon, breast, stomach, and skin cancer models. In addition, Oltipraz has been shown to be non-mutagenic, a radioprotector, and a chemoprotective agent against carbon tetrachloride and acetaminophen toxicity (*119*).

Oltipraz protects against AFB_1- induced hepatocarcinogenesis in rats when fed before and during carcinogen exposure; however, such an exposure chemoprotection is not directly relevant to most human populations. GST catalyzes the detoxication of aflatoxin-8,9-epoxide and was found to be rapidly induced in the livers of animals after the beginning of the Oltipraz intervention. The significant protection against presumptive pre-neoplastic tumors, suggests that Oltipraz may exert substantial activity against the cytotoxic and autopromoting action of repeated exposures to AFB_1 and supports the utility of intervention trials with Oltipraz in individuals chronically consuming AFB_1-contaminated foods, particularly in regions with high incidences of liver cancer (*120*). Oltipraz is reported to be useful for the modulation of gene expression in subjects at risk for colorectal cancer (*121*).

Cruciferous vegetables, such as broccoli, contain anticarcinogens. Glucoraphanin, the principal glucosinolate in broccoli sprouts, can be hydrolyzed by gut microflora to sulforaphane, a potent inducer of carcinogen detoxication enzymes. A randomized, placebo-controlled chemoprevention trial tested whether drinking hot water infusions of 3-day-old broccoli sprouts, containing defined concentrations of glucosinolates, could alter the disposition of AF and phenanthrene. An inverse association was observed for excretion of AF-DNA adducts in individuals receiving broccoli sprout glucosinolates. Effects of glucosinolate-rich broccoli sprouts on urinary levels of aflatoxin-DNA adducts and phenanthrene tetraols were studied in a randomized clinical trial in He Zuo township, Qidong, People's Republic of China (*122, 123*).

Indole-3-carbinol (I3C), a component of cruciferous vegetables, in experimental diets inhibited *in vivo* AFB_1-DNA adduction by 68% and combined with BNF the inhibition was 51%) (*108*). AFB_1-induced tumors or pre-neoplastic lesions in experimental animals can be inhibited by co-treatment with several compounds, including I3C and the well-known Ah receptor agonist BNF. This study examined the influence of these two agents on the AFB_1-glutathione detoxication pathway and AFB_1-DNA adduction in rat liver (*108*).

Chemoprevention of AFB_1 hepatocarcinogenesis by coumarin, a natural benzopyrone that is a potent inducer of AFB_1-aldehyde reductase, the glutathione S-transferase A5 and P1 subunits, and NAD(P)H:quinone oxidoreductase in rat liver, was studied (*124*). The consumption of a coumarin-containing diet provides substantial protection against the initiation of AFB_1 hepatocarcinogenesis in the rat (*124*). There are some phytochemicals (benzyl isothiocyanate, coumarin, or indole-3-carbinol), synthetic antioxidants, and other drugs (butylated hydroxyanisole, diethyl maleate, ethoxyquin, beta-naphthoflavone, Oltipraz, phenobarbital, or *trans*-stilbene oxide) that increase hepatic aldo-keto reductase activity toward AFB_1-dialdehyde and GST activity toward AFB_1-8,9-epoxide in both male and female rats.

Ellagic acid (EA), a plant phenol found in various fruits and nuts, was examined for its ability to inhibit AFB_1 mutagenesis in strain TA-100 of *Salmonella typhimurium*. Inhibition of AFB_1 mutagenesis in *S. typhimurium* and DNA damage in cultured rat and human tracheobronchial tissues by ellagic acid was reported (*101*). In the presence of rat liver S_9 microsomal preparation, EA (1.5 μg /plate) inhibited the number of mutations induced by AFB_1 (0.5 μg/plate) by 50%. EA at a dose of 1000 μg/plate inhibited the mutation frequency by greater than 90%. In tissues, the major AFB_1-DNA adducts were AFB_1-N^7-Gua [8,9-dihydro-8-(N^7-guanyl)-9-hydroxy-AFB_1] and AFB_1-N^7-FaPyr (major) [8,9-dihydro-8-(2,6-diamino-4-oxo-3,4-dihydropyrimid-5-yl-formamido)-9-hydroxy-AFB_1]; the formation of these adducts was reduced by 28-76% in the presence of ellagic acid. These data indicate that ellagic acid has the potential to act as a naturally occurring inhibitor of AFB_1-related respiratory damage in rats and in humans (*101*).

The diterpenes cafestol and kahweol (C and K) have been identified in animal models as two potentially chemoprotective agents present in green and roasted coffee beans. Cavin *et al.* (*125*) postulated that these compounds may act as blocking agents by producing a co-ordinated modulation of multiple enzymes involved in carcinogen detoxification. Significant inhibition was detected at 2300 mg/kg and maximal reduction of DNA adduct formation to nearly 50% of the control value was achieved with 6200 mg/kg of dietary C and K. Two complementary mechanisms may account for the chemopreventive action of cafestol and kahweol against AFB_1 in rats. A decrease in the expression of the rat activating cytochrome P_{450}s (CYP2C11 and CYP3A2) was observed, as well as a strong induction of the expression of the GST subunit GST Yc2, which is known to highly detoxify the most genotoxic metabolite of AFB_1. These data suggest the potential widespread effect of these coffee components against chemical carcinogenesis (*125*).

Other authors refer to potent protection against AF-induced tumorigenesis through induction of Nrf2-regulated pathways by the triterpenoid 1-[2-cyano-3,12-dioxooleana-1,9(11)-dien-28-oyl] imidazole (*126*). Certain monoterpenes reduced formation of adducts in rats exposed to AFB_1 (*127*).

Influence of grapefruit juice intake on AFB_1-induced liver DNA damage was examined using a Comet assay in F344 rats given 5 mg/kg AFB_1 by gavage. Grapefruit juice extract inhibited AFB_1-induced mutagenesis in the presence of a microsomal activation system from livers of humans as well as rats. Grapefruit juice intake suppresses AFB_1-induced liver DNA damage through inactivation of the metabolic activation potency for AFB_1 in rat liver (*128*).

With respect to vitamins the effects of carotenoids on the initiation of liver carcinogenesis by AFB_1, male weanling rats were fed beta-carotene, beta-apo-8'-carotenal, canthaxanthin, astaxanthin, or lycopene (300 mg/kg diet), or an excess of vitamin A (21,000 RE/kg diet), before and during i.p. treatment with AFB_1 (2 x 1 mg/kg body wt) (*58*). In contrast to lycopene or to an excess of vitamin A, both of which had no effect, *beta*-carotene, *beta*-apo-8'-carotenal, astaxanthin and canthaxanthin, as well as 3-MC, were very efficient in reducing the number and the size of liver preneoplastic foci. In a similar way to 3-MC, the $P_{450}1A$-inducer carotenoids, *beta*-apo-8'-carotenal, astaxanthin, and canthaxanthin decreased *in vivo* AFB_1-induced DNA SSB and the binding of AFB_1 to liver DNA and plasma albumin, and increased *in vitro* AFB_1 metabolism to AFM_1, a less genotoxic metabolite. These carotenoids exert their protective effect through the deviation of AFB_1 metabolism towards detoxication pathways. In contrast, beta-carotene did not protect hepatic DNA from AFB_1-induced alterations, and caused only minor changes of AFB_1 metabolism: seemingly, its protective effect against the initiation of liver preneoplastic foci by AFB_1 is mediated by other mechanisms (*58*). On the other hand, there are reports about the protective effect of ascorbic acid in guinea pigs with acute aflatoxin toxicity (*129*). The results indicated that intake of 300 mg of ascorbic acid almost protected the animals from acute toxicity of AFB_1 when given by gavage, but not when administered as a second dose i.p. (*129*).

Oldenlandia diffusa and *Scutellaria barbata* have been used in traditional Chinese medicine for treating liver, lung and rectal tumours. Wong *et al.*(*130*) showed that they inhibited mutagenesis, DNA binding, and metabolism of AFB_1 bioactivated by Aroclor 1254-induced rat S_9. *O. diffusa* and *S. barbata* consistently inhibited the mutagenicity of AFB_1 bioactivated by either non-induced or dexamethasone-induced S_9. *O. diffusa* and *S. barbata* may possess antimutagenic and antitumorigenic activity towards AFB_1 through an inhibition of CYP3-mediated metabolism of AFB_1 (*130*).

Chlorophyllin is another natural product that has been reported as useful to reduce aflatoxin-DNA adducts in individuals at high risk for liver cancer (*131*).

In vitro and *in vivo* studies suggest that selected strains of probiotic bacteria can form tight complexes with AFB_1 and other carcinogens. The administration of probiotic bacteria could block the intestinal absorption of AFB_1 and thereby lead to reduced urinary excretion of AFB_1-N^7-guanine, a marker for a biologically effective dose of AF exposure. Elevated urinary excretion of this AF-DNA adduct is associated with an increased risk of liver cancer. A probiotic supplement reduces the biologically effective dose of AF exposure and may thereby offer an effective dietary approach to decrease the risk of liver cancer (*132*).

Finally, human hepatocytes (HepG2) cells pretreated with lycopene and beta-carotene are protected from the toxic effects of AFB_1 at both the cellular and molecular levels (*133*).

The information presented here covers just some aspects of adducts that are interesting for scientists of other specialities.

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Chapter 3

Combined Toxic Effects of Ochratoxin A and Citrinin, In Vivo and In Vitro

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Ochratoxin A (OTA) and citrinin (CIT), which are nephrotoxic mycotoxins suspected to be involved in Balkan endemic nephropathy and urothelial tract tumours, are known to co-exist in certain food sources, such as cereals. Simultaneous administration of OTA and CIT enhanced the incidence of renal tumors in male mice. The aim of this study was to determine the combined genotoxic effects of CIT and OTA, (i) in cell culture and (ii) *in vivo* in Dark Agouty rats fed for 3 weeks with ground wheat enriched with OTA and/or CIT. When the mycotoxins are simultaneously present, the toxicity was considerably enhanced. DNA adduct patterns of rat kidney after a 3-weeks feeding, were similar to those obtained in cell cultures. The main OTA DNA-adduct was identified as C8 dGMP-OTA, which was found in human tumors, and found to increase by simultaneous presence of CIT and OTA.

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Cereals and other crops are susceptible to fungal attack either in the field or during storage.These fungi may produce mycotoxins. During storage, Aspergillii (*Aspergillus ochraceus, A. carbonarius, A. niger*) and Penicillia (*Penicillium verrucosum, P. griseofulvum P. citrinum, and P. expansum*) produce ochratoxin A (OTA) and citrinin (CIT). OTA is nephrotoxic and to date is one of the most potent renal carcinogens studied by the National Cancer Institute/National Toxicological Program (NCI/NTP) (*1*, for a review see *2*). OTA is associated with the fatal human kidney disease, Balkan endemic nephropathy (BEN) (*3, 4*). Balkan endemic nephropathy (BEN) is a familial chronic tubulointerstitial disease with insidious onset and slow progression to terminal renal failure. It was first described in Serbia and in Bulgaria. It affects people living in the alluvial plains along the tributaries of the Danube River in Serbia, Bosnia, Croatia, Bulgaria, and Romania. The disease usually affects adults in their fourth/fifth decade with eventual end-stage renal failure in their sixth decade. An association between BEN and urinary tract tumors (UTT) was recognised in the three affected countries (for reviews see *2, 4-6*). In 1972, on the basis of a series of epidemiological observations, Akhmeteli (*7*) suggested that fungal toxins could be involved in the etiology of BEN. Nikolić *et al.* (*8*) demonstrated that the very intimate link between BEN and UTT can be explained by insult from an environmental contaminant. BEN affects inhabitants in rural areas but not those from towns in the vicinity. This could be explained by the fact that rural populations consumed homegrown and home-stored food, while urban populations consumed commercial foods produced by factories. A study of food contamination conducted in Bulgaria demonstrated that a higher percentage of the staple food (maize and beans) was contaminated by OTA in the endemic area than in the non-endemic area (*9, 10*). Re-analysis of the data (*4*) showed a striking difference, demonstrating that, in Bulgaria, affected families are not only much more frequently exposed to the mycotoxins OTA and CIT than the control families but also to higher amounts of both toxins. The amount of CIT was often ten times higher in beans or maize from affected families compared to those of non-affected families. Vrabcheva *et al.* (*11*) found that in the BEN endemic area, wheat samples could also be contaminated by OTA, and at higher levels than in the control non-endemic region. Higher exposure levels to OTA have been confirmed in the BEN households than in the within-village controls and in the controls in BEN-free villages (*12*). Experimental ochratoxicosis in pigs has sought to define the role of ochratoxin A in natural occurrence of porcine nephropathy in pig production, notably and classically in Denmark (*13*), but also in Bulgaria (*14*). Its primary role as causal agent in the former was well recognised, if also occasionally in conjunction with other natural mycotoxins in feed. It is very likely that humans and animals are always exposed to mixtures of mycotoxins rather than to individual compounds. Therefore studies of the effects of mycotoxins in combination are very relevant in order to ascertain whether toxins interact with other toxins, which will provide for an improved and more realistic risk assessment. *In vivo* studies have shown synergistic lethal effects of these two mycotoxins in mice (*1, 15*), dogs (*16*), guinea pigs (*17*), and neonatal rats (*18*). Simultaneous administration of OTA (25 mg/kg) and CIT (200 mg/kg) enhanced the incidence of renal cell tumors in male DDD mice induced by OTA alone; CIT alone was not carcinogenic (*19*). More recently Jeswal (*20*) observed also an increase of renal tumors in mice orally treated simulatenously with 50 µM of OTA and 50 µM of CIT.

The aim of this study was to evaluate the contamination of French wheat by OTA and CIT and to determine the (geno)toxic combined effect of CIT and OTA, (i) in cell culture and (ii) *in vivo* in Dark Agouty rats fed for 3 weeks with ground wheat enriched with OTA and/or CIT.

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Material and Methods

Materials

OTA (benzene free, CAS# 303-47-9); CIT (CAS# 518-75-2) and the following enzymes: proteinase K (used as received), RNase A, RNase T1 (boiled 10 min at 100 °C to destroy DNases), and microccocal nuclease (dialyzed against deionized water) were from Sigma (Saint Quentin Fallavier, France); spleen phosphodiesterase (centrifuged before use) was from Calbiochem (VWR, France), nuclease P_1 (NP_1) and T4 polynucleotide kinase were from Roche Diagnostics (Meylan, France). [γ ^{32}P-ATP] (444 Tbq/mmol, 6000 Ci/mmol) was from Amersham (Les Ullis, France); Dubelco's Eagle's minimum essential medium (D-EMEM) and Roswell Park Memorial Institute medium (RPMI) were prepared with Gibco products (Cergy Pontoise, France); phosphate saline buffer, trypsin, fetal calf serum, streptomycin and penicillin were from Life-Technologies (Cergy-Pontoise, France); rotiphenol (phenol saturated with TRIS-HCl, pH 8) was from Rothsichel (Lauterbourg, France); cellulose MN 301 was from Macherey Nagel (Düren, Germany); polyethyleneimine (PEI) was from Corcat (Virginia Chemicals, Portsmouth, VA, U.S.A.); Whatman No. 1 paper (ref. 6130932) was from VWR (France) and PEI/cellulose TLC plates used for 32P-postlabeling analyses were prepared in the laboratory at Toulouse, France. All reagents (potassium chloride, sodium hydrogen carbonate, sulfuric acid, phosphoric acid, hydrochloric acid, acetic acid, sodium dihydrogen phosphate) were of normal grade. C-C8dGMP-OTA standard was synthesized at the University of Guelph as described in Faucet *et al.* (*21*).

Experimental Procedures

Cell Culture

Human bronchial epithelial cells (WI26; ATCC CCL-95.1) and human kidney cells (HK2; CRL-2190) were provided by ATCC (American Type Culture Collection, Manassas, VA, USA). WI cells were cultured in RPMI and HK2 in D-EMEM medium containing 44 mM $NaHCO_3$, 5 % fetal bovine serum (FBS), 2% vitamins, 2% of non-essential amino acids, 1% streptomycin, and 1% penicillin, for 48 h at 37 °C under 5% CO2. After trypsin digestion, the cells were re-suspended in this medium to obtain 1x 10^6 cells per mL.

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In vivo *Study*

Dark Agouti male rats, average six weeks old, weighing 80 g were housed in individual cages (43x21.5x20 cm) and kept in environmentally controlled conditions (ventilation, 22 °C, 12 h dark/light cycles). After a four day acclimation period, they were randomly caged by 5 animals per group and were fed during 21 days with standard AIN 76® pellet (UAR, Villemoisson, France), contaminated or not with either OTA (26 µg/kg feed) or CIT (100 µg/kg feed) or both. During the study, food consumption, weight gain, and physiological parameters were checked. Urine samples were collected once a week until the end of the study. On completion of the treatment period, animals were killed and organs were frozen at –80 °C. At the necropsy, blood samples (approximately 5 mL) were collected, from the abdominal aorta, into EDTA tubes and used for quantification of OTA in total blood.

Isolation of DNA

Cell pellets were homogenized in 0.8 mL of a solution containing NaCl (0.1 M), EDTA (20 mM), and Tris-HCl, pH 8 (50 mM) (SET) in an ice bath. To the homogenate, 100 µL of a 20% solution of sodium dodecylsulfate was added, and following incubation for 10 min at 65 °C, 800 *µ*L of potassium acetate (6M, pH 5) was added. The reaction mixture was kept at 0 °C for 30 min. After centrifugation for 25 min at 0 °C (10,000*g*), the supernatant, which contained nucleic acids, was collected and nucleic acids were precipitated overnight at -20 °C by adding 2 volumes of cold ethanol. The DNA pellets were collected and washed once with 1 mL of 90% ethanol and dissolved in 500 µL of SET (15 min at 37 °C). The total extract was mixed with 10 µL of a mixture of RNase A (20 mg/mL) and RNase T1 (10,000 U/mL) and incubated for 1 h at 37 °C; this treatment was repeated twice. Samples were then treated with 25 *µ*L of proteinase K solution (20 mg/mL SET) for 1 h at 37 °C. After digestion, 500 μL of rotiphenol® was added and the mixture was moderately shaken for 20 min at room temperature and centrifuged for 15 min at 15 °C (10,000 *g*). The aqueous phase was collected after two extractions. After a final extraction with one volume of chloroform-isoamyl alcohol (24:1, v/v), the aqueous phase was collected and 50 μL of sodium acetate (3 M, pH 6) was added. The DNA was precipitated by the addition of two volumes of cold ethanol overnight at -20 °C, and the precipitate was collected by centrifugation at 10,000 *g* for 30 min. The DNA pellet was washed four times with 90% ethanol. DNA was dissolved in deionized water and tested for purity by UV-vis spectroscopy.

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^{32}P-postlabeling Analysis of DNA Adducts

The equivalent of 4 μg DNA were dried in vacuum, dissolved in 10 μL of the mix containing 1 μL of micrococcal nuclease (2 mg/mL corresponding to 500 U), spleen phosphodiesterase (15 mU/μg DNA), 1 μL of sodium succinate (200 mM), and 1 μL of calcium chloride (100 mM, pH 6) and digested at 37 °C for 4 h. The digested DNA was then treated with 5 μL of the mix containing 1.5 μL of NP1 (4 mg/mL), 1.6 μL of $ZnCl_2$ (1 mM), and 1.6 μL of sodium acetate (0.5 M, pH 5) at 37 °C for 45 min. The reaction was stopped by addition of 3 μL of Tris base (500 mM). The DNA adducts were labelled as follows: to the NP1 digest, 5 μL of the reaction mixture containing 2 μL of bicine buffer [Bicine (800 μM), dithiothreitol (400 mM), $MgCl_2$ (400 mM), and spermidine (400 mM) adjusted to pH 9.8 with NaOH], 10 U of polynucleotide kinase T_4, and 100μCi of [γ-^{32}P]ATP (specific activity 6000 Ci/mmol) were added and the mixture incubated at 37 °C for 45 min. Normal nucleotides, pyrophosphate, and excess ATP were removed by chromatography on PEI/cellulose TLC plates (D1) in 3 M NaH_2PO_4 buffer pH 5.7 overnight. The origin (4 cm) areas containing labelled adducted nucleotides were cut out and transferred to another PEI/cellulose TLC plate, which was run (D2) for 3 h in 4.8 M lithium formate and 7.7 M urea, pH 3.5. A further (D3) migration was performed after turning the plate 90° anticlockwise in 1 M NaH_2PO4 and 4.5 M urea, pH 6.4, for 3 h. Finally, the chromatogram was washed in the same direction in 1.7 M NaH_2PO_4, pH 6, for 2 h (D4). Adduct profiles were analyzed qualitatively and semi-quantitatively by autoradiography of the plates, carried out at -80 °C for 48 h in the presence of an intensifying screen, using a radio-analytical system of image analysis (AMBIS, Lablogic).

Cytotoxicity Evaluation

Cytotoxicity was determined using the MTT test. Cell titer 96® non-radioactive cell proliferation assay (Promega) is based on the cellular conversion of a tetrazolium salt into a yellow formazan product. The amount of color formed is directly proportional to the number of viable cells and is detected using a 96 plate reader at 490 nm.

Determination of OTA and CIT Content

OTA and CIT were extracted from the wheat, the feed, the tissues, the blood, and the urine as described by Pfohl-Leszkowicz *et al.* (*22*) and Molinié *et al.* (*23*). Briefly, 25 g ground wheat sample was extracted, under agitation, with 200 mL of acetonitrile-water (9:1, v/v), water containing 4% KCl and 0.4 mL of pure sulfuric acid. After filtration, a 100 mL aliquot was defatted twice with 50 mL *n*-hexane. Following addition of 25 mL water, the acetonitrile-water phase was extracted successively three times with chloroform (50, 10, 10 mL). The combined chloroform extracts were partitioned three times against 25 mL of 5% sodium hydrogen bicarbonate.The combined aqueous phases were acidified to pH 1.5 with hydrochloric acid and re-extracted three times with chloroform (50, 25, 25 mL). The combined chloroform extract was dried under vacuum, at 45 °C. The dried residue was dissolved in 1 mL methanol, filtered through a 0.2 µm filter, and dried under nitrogen flow. Finally, the filtrate was dissolved in 500 µL methanol and kept at -20 °C.

OTA was extracted from tissue, blood, serum and urine as described by Petkova-Bocharova *et al.*. (*24*). Tissue (1 g) was blended with 20 mL $MgCl_2$ 0.1M/HCl 0.05 M, pH 1.5, and extracted three times with chloroform (20, 10, 10 mL). Combined chloroform extracts, obtained after centrifugation (10 min, 5000 rpm, 4 °C) were partitioned twice against 0.1 M sodium hydrogen carbonate (20 mL). The aqueous phase was acidified to pH 1.5 and extracted three times with chloroform (20 mL). The combined chloroform extracts were dried under vacuum, dissolved in 1 mL methanol, filtered, dried under nitrogen and finally dissolved in 200 µl methanol. The same protocol was applied to 2 mL urine, 1 mL blood and 500 µl plasma, using 8 mL 0.1 M $MgCl_2$, 0.05 M HCl, pH 1.5, and 8 mL chloroform for extraction, and 16 mL sodium hydrogen carbonate and 16 mL chloroform for partition.

LC Analyses

OTA was determined by reversed phase LC using a 15 cm Nucleosil 100-3-C18 column, with isocratic elution (600 mL methanol, 600 mL acetonitrile, 800 mL ultra pure water, 0.68 g sodium acetate. 3 H_2O, and 28 mL glacial acetic acid,). Detection was performed with a programmable fluorimeter GTI spectrovision (excitation 340 nm, emission 465 nm).

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CIT was analysed by reversed phase LC using the same column as for OTA and isocratic elution (700 mL (0.33 M H_3PO_4 300 mL acetonitrile, and 50 mL propan-2-ol,. Detection was performed by fluorimetry (excitation 331 nm, emission 550 nm) (*23*)

Solvents were HPLC grade and columns were from ICS (Lapeyrouse-Fossat, France).

Treatment of data: Comparison between groups was done by comparison of medians using the software SPSS 12.0, based on the statistical analysis described by McGill *et al.* (*19*). Differences between groups are statistically significant when the median of the treated group is two fold higher than the control group and the standard deviations (SD) do not cross over. A trend of significance is observed when medians are different (less than two-fold), but the SD do not cross over.

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Wheat Sampling

From July 2001 to June 2002, 83 wheat samples were collected from 12 different storage sites belonging to cooperatives in Midi-Pyrénées (southwest of France). Some samples (28) were collected immediately after harvest, without storage; 41 samples were collected at different dates during storage; 14 were stored on-farm for several months before storage in the cooperative.

Results

Wheat Contamination

More than a half of the wheat samples collected in France contained traces of OTA and/or CIT ranging from the limit of quantification (LOQ 0.2 µg/kg) to 11.6 µg/kg for OTA; and from LOQ to 6 µg/kg for CIT (Figure 1). OTA was the main contaminant (78 % of the contaminated samples). Only 12 % of the contaminated samples contained OTA over 5 µg/kg (Table I).

Twenty four % of the contaminated samples contained OTA and CIT simultaneously. Figure 2 shows the respective concentrations of OTA and CIT. The co-contaminated samples were more often stored on-farm (7/12). Harvesting after a rainy period also increased the risk of co-contamination and the level of contamination. In some cases the amounts of both toxins were similar; in other cases the amount of CIT was higher (2-6 fold); sometimes the amount of OTA was higher than that of CIT (up to 3 times).

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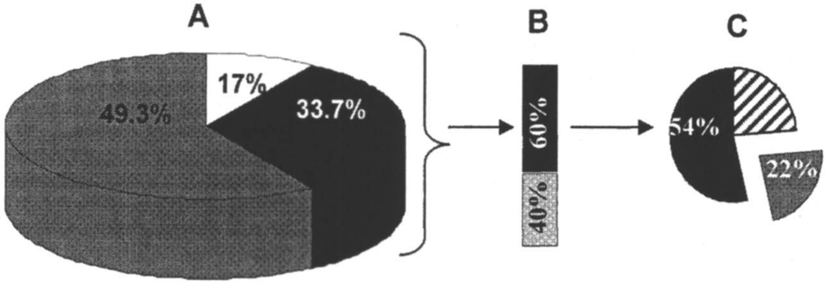

Figure 1. OTA or CIT analyses of French wheat samples: [A] type of samples analysed, (black) unstored sample; (grey) stored in grain storage cooperative; (white) stored on-farm before cooperative; [B](black) percent of samples contaminated by either OTA or CIT; (grey) not contaminated; [C] mycotoxin repartition, (black) contaminated only by OTA ; (grey) only by CIT; (hatched) OTA + CIT.

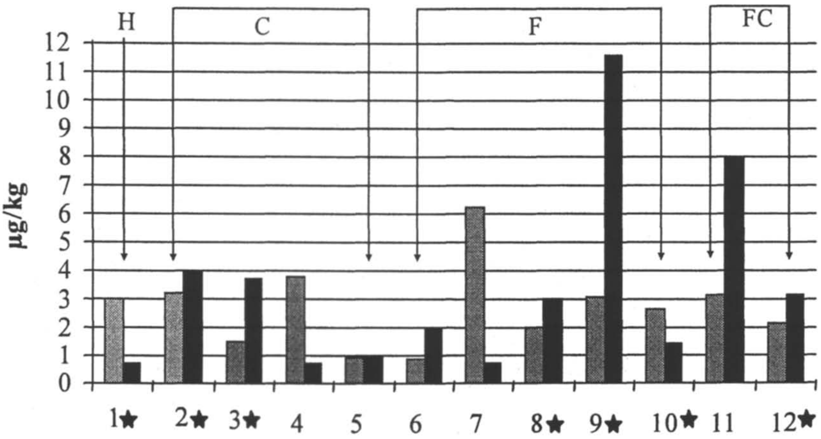

Figure 2. Concentrations of OTA (black) and CIT (grey) in sample co-contaminated; H, sample collected during harvest; C, samples stored in cooperative; F, samples stored on-farm; FC, samples stored first on-farm and then in cooperative; ★ samples harvested after a rainy period.

Table I. Range of OTA and CIT Contamination

OTA range	*Number of Samples*	*% Contaminated (n=39)*	*% of Total (n= 83)*
LOQ< OTA < 3 μg/kg	27	70	32
3 < OTA< 5 μg/kg	7	18	8.4
> 5 μg/kg	5	12	6

CIT range	*Number of Samples*	*% Contaminated (n=25)*	*% of Total (n=83)*
LOQ < CIT < 5 μg/kg	19	83	23
> 5 μg/kg	4	17	4

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Combined Effect of OTA and CIT on Cell Viability

Kidney cells were treated with increasing amounts of OTA (0.5 μM to 50 μM) alone or in the presence of increasing amounts of CIT (0.5 μM to 50 μM). OTA was more cytotoxic than CIT. Depending on the relative proportion of OTA and CIT an antagonism or a synergy was observed (Figure 3). When the concentration of CIT was lower than that of OTA, the cytotoxic effect was lower compared to the cytotoxic effect induced by the same concentration of OTA alone. On the contary, when the concentration of CIT was equal or higher than the concentration of OTA the cytotoxic effect was increased.

Animal Study

A statistical decrease of body gain weight was observed in the group of animals fed exclusively with feed contaminated with OTA alone compared to animals fed a normal diet (Figure 4). Only a slight trend of decrease was observed when animals were fed with CIT alone, whereas no difference between control animals and animals fed with both toxins was observed.

No CIT was detected in the blood of animals fed with a diet contaminated with CIT only whereas 1.1 ng/mL of CIT was observed in animals fed with both toxins. Less OTA was found in the blood of animals fed with both toxins (39.4 ng/mL) compared to animals fed with diet containing the same amount of OTA alone (53.9 ng/mL). OTA and CIT were found in larger concentrations in kidney than in liver, three and five times respectively for OTA and CIT. In the kidney and the liver of animals fed with diet containing both toxins, less OTA and CIT were found either in kidney or in liver (Figure 5). Elimination of OTA via urine was very limited. In animals fed with OTA and CIT, the elimination of both toxins was increased (Figure 6). This elimination was very important in the first

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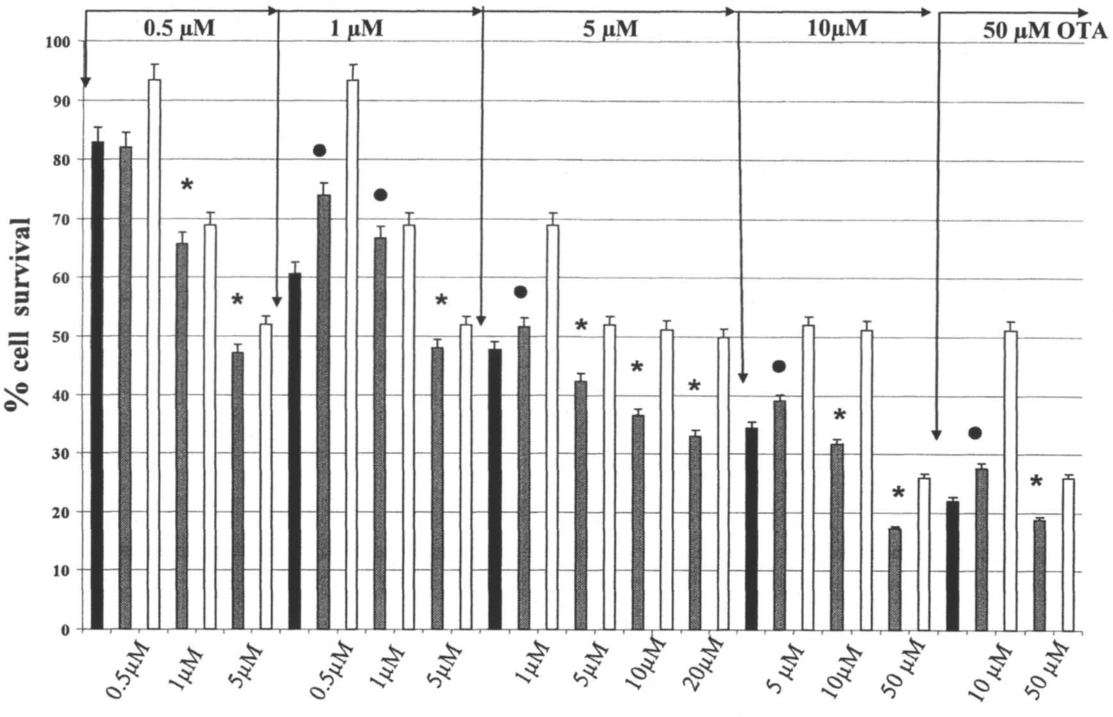

*Figure 3. Cytotoxic effect of OTA combined or not with CIT on opposum kidney cells; (black) increasing concentrations of OTA alone; (white) increasing concentrations of CIT alone; (grey) OTA + CIT; * additive effect; ● decrease of cytotoxicty compared to OTA alone.*

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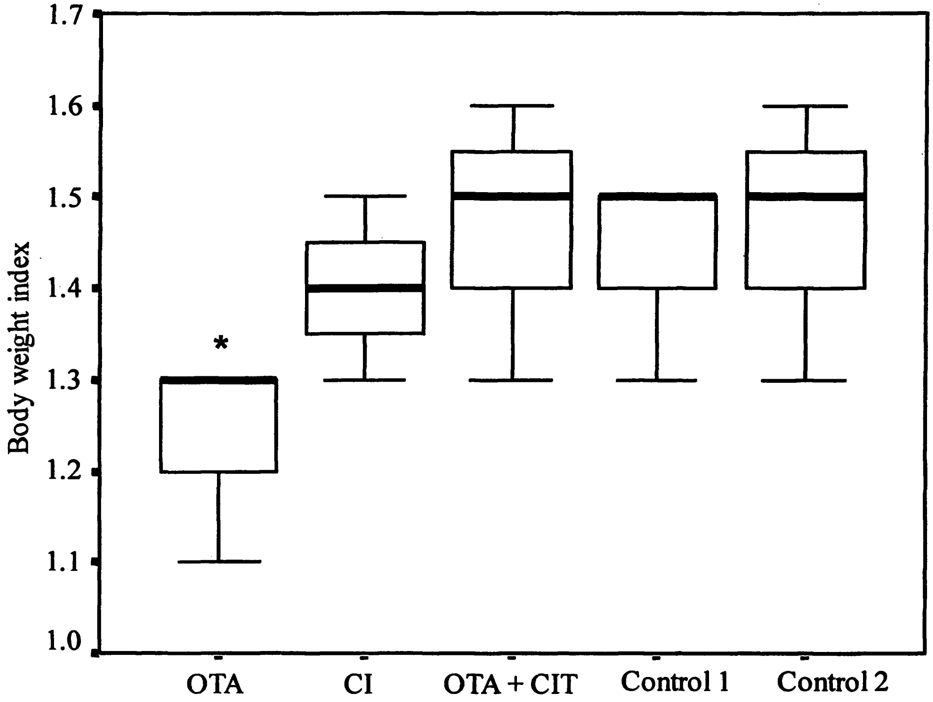

Figure 4. Body weight gain of rats fed three weeks with feed contaminated by OTA and/or CIT.
** significant decrease compared to control animals.*

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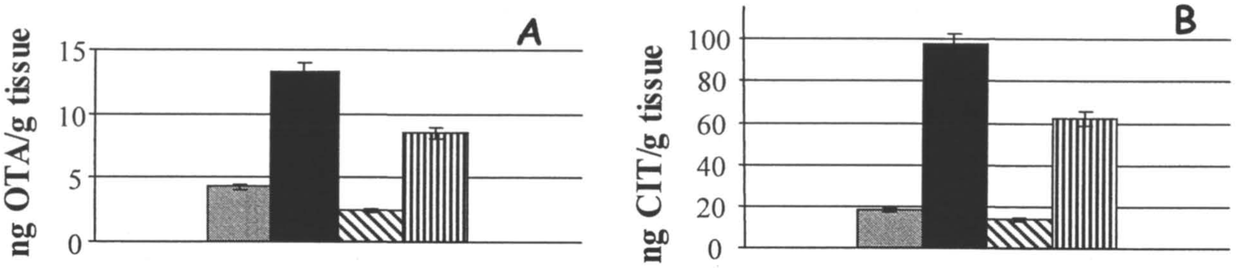

Figure 5. Concentrations of OTA [A] and CIT [B] in kidney (grey and obliquely hatched) and in liver (black and horizontally hatched).

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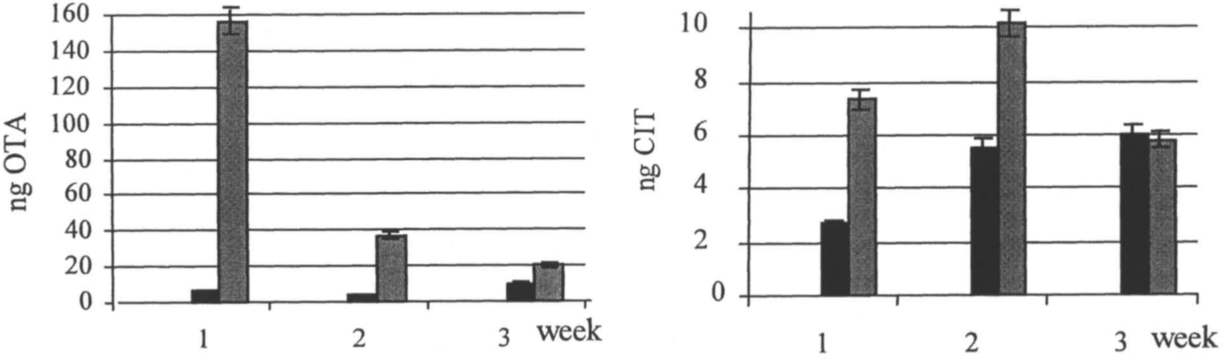

Figure 6. Amount of OTA [A] or CIT [B] eliminated via the urine by rats fed OTA or CIT alone (black); OTA and CIT (grey).

week of feeding OTA. There was no direct correlation between intake, tissue accumulation, and elimination of the toxins (Figure 7). Although CIT intake was four fold higher than that of OTA a larger proportion of CIT was stored in the kidney (7 times more), whereas almost no CIT was found in blood, and an equivalent amount of CIT and OTA was excreted via urine.

DNA Adduct Formation

Examples of kidney DNA adduct patterns of rats fed with diet containing either OTA alone or CIT alone or both together are given in Figure 8. No DNA adduct was observed in kidney of animals fed with normal feed (Figure 8A). Mainly two adducts were formed in the kidney of animals fed OTA (Figure 8B). CIT also induced several adducts in the rat kidney (Figure 8C). In kidney of animals fed with diet containing both toxins, the major specific adduct related to OTA was considerably increased (by 10 times) (Figure 8D), whereas the formation of the specific adducts related to CIT were almost completely inhibited. Formation of DNA adduct has been tested in cell culture (Figure 9). Treatment of cells with OTA induced the same two adducts as *in vivo* (Figure 9B). In the same way treatment with CIT induced the formation of three main adducts similar to those observed *in vivo* (Figure 9C). Co-treatment with OTA and CIT increased the major adduct formed by OTA (Figure 9D). This adduct has the same chromatographic properties as the C-C8dGMP OTA adduct (Figure 9E). The formation of CIT-specific DNA adduct was dose- and time-dependent (Figure 10). After 12 h of treatment, 1 µM of CIT did not induce DNA adduct formation, whereas 10 and 50 µM induced increasing amounts of DNA adduct (Figure 10A). When the cells were treated with 50 µM of CIT, DNA adduct increased, reached a maximum after 16 h, and completely disappeared after three days (Figure 10B).

Discussion

Analysis of wheat samples indicated that CIT was frequently found as a co-contaminant of OTA. In general, although half of the samples had trace levels of CIT and OTA, the concentration of OTA was much below the EU limit of 5 µg/kg. Samples stored on-farm and harvested after a rainy period were more contaminated than the others, confirming the data obtained previously (for a review see Pfohl-Leszkowicz and Manderville (*2*).

Cytotoxic effects of OTA, CIT, or OTA + CIT were measured using kidney cells and the mixture consistently showed greater cytotoxicity than OTA alone. These data are in line with those of other authors who observed a cytotoxic effect of OTA when NIH/3T3 cells were treated with 25 µM (*25*), when LLC-

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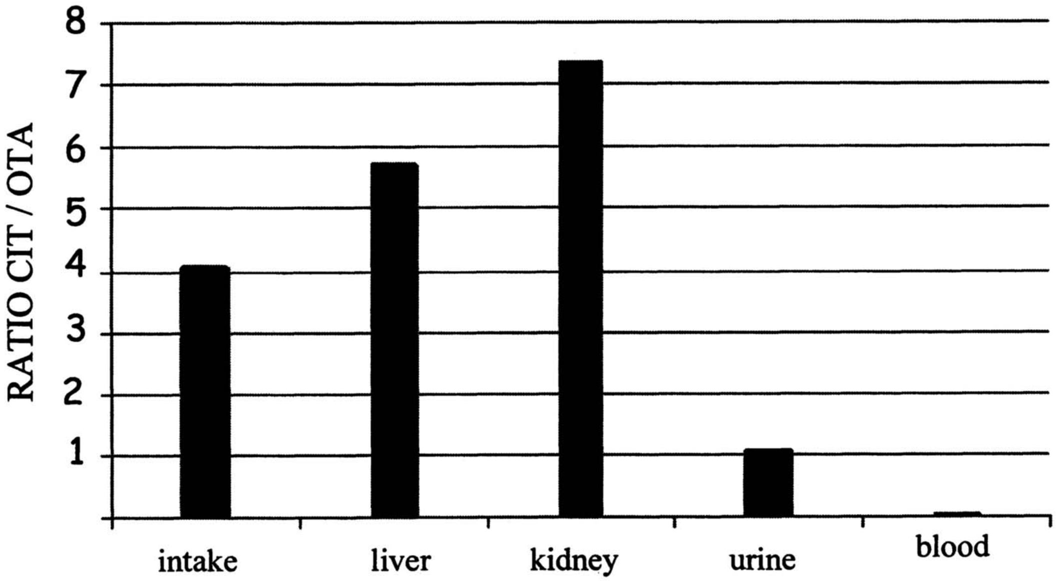

Figure 7. Relative proportion of CIT and OTA in the different compartments of rats fed OTA and CIT.

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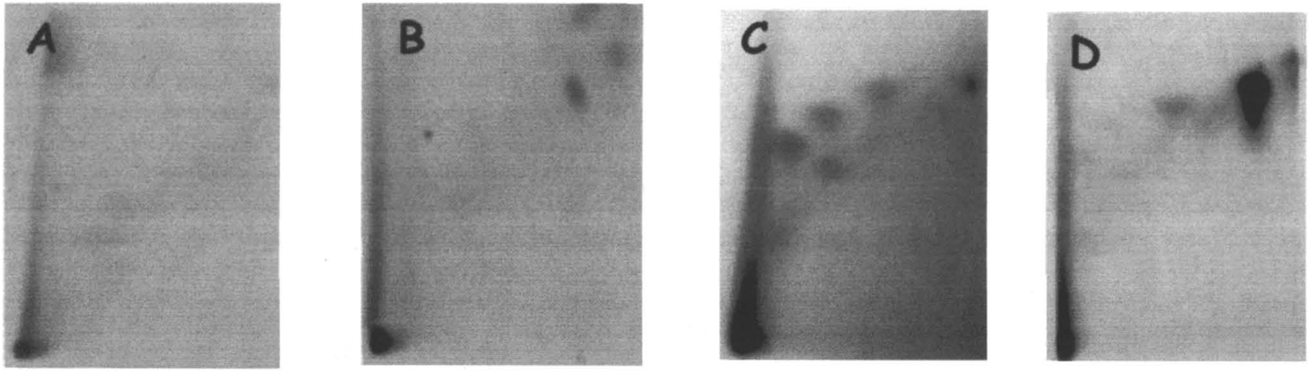

Figure 8. Kidney DNA adduct pattern of rat fed with: [A] non-contaminated feed; [B] OTA alone; [C] CIT alone; [D] OTA + CIT.

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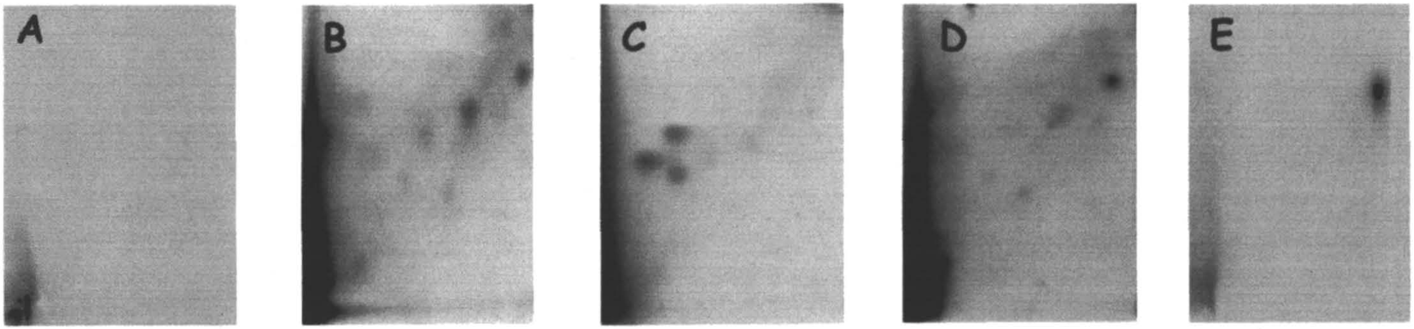

Figure 9. DNA adduct pattern of cell treated by OTA or CIT [A] control cell; [B] 10 μM OTA alone; [C] 50 μM CIT alone;[D] 10 μM OTA + 50 μM CIT; [E] C-C8dG OTA standard.

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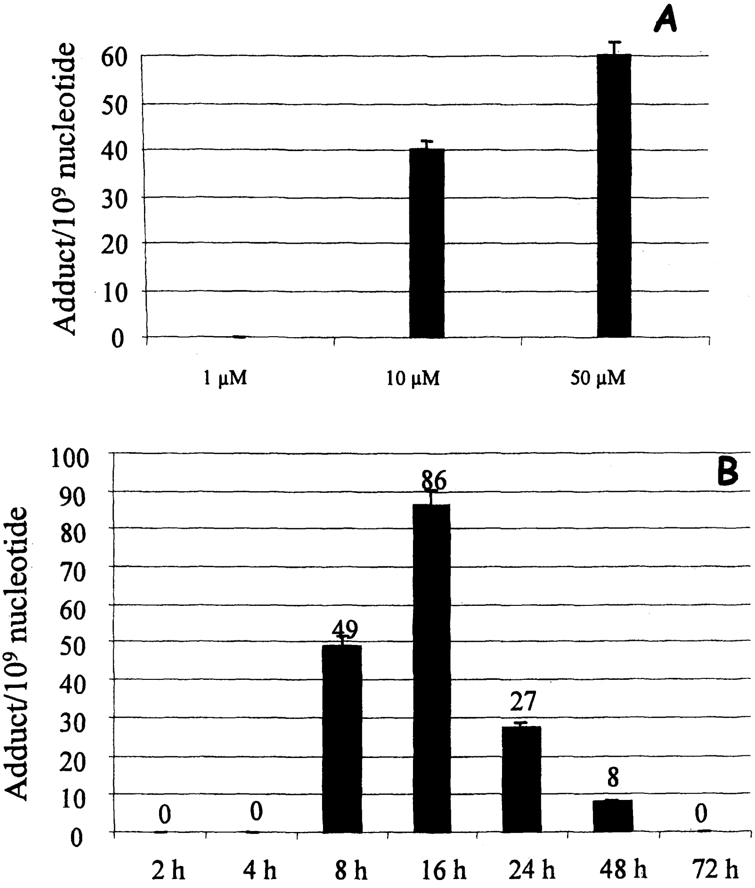

Figure 10. Dose [A] and time [B] dependent DNA adduct formation in cell treated by CIT.

PK1 cells were treated with 12.5 µM (*26*), and when WB-F344 or SB3 cells were treated with 80 µM (*27*). In the same way, 16 µM and 25 µM of OTA induced cytotoxicity in primary cell cultures of proximal tubular kidney cells from pig or rat, respectively (*26, 28*). Our cells are more sensitive as we observed a cytotoxic effect after treatment with 1 µM of OTA. The cytotoxic effect of CIT was also in the same range as observed by others. A recent study showed that the viability of HEK 293 cells (human embryonic kidney cell) treated with 80 µM for 48 h decreased to 60% (*29*). Increase of the cytotoxic effect of OTA by CIT could be due to the ability of CIT to modify the expression of biotransforming enzyme (*30*), notably CYP 2C9 known to favor the cytotoxicity of OTA (*25*) and the genotoxicity (*31*). More recent studies have shown that OTA and CIT elicit a synergistic effect on mitogen induced lymphocyte proliferation (*32*). In this study lymphocyte proliferation by *Penicillium* mycotoxins, including CIT, cyclopiazonic acid (CPA), OTA, patulin (PAT), penicillic acid (PIA), and roquefortine (RQC) was studied using purified lymphocytes from six piglets. Only one of the 15 pairs of toxins, OTA + CIT, elicited a statistically significant synergistic effect (*32*). Combined cytotoxicity of OTA and other mycotoxins in renal cells (*33*) also showed interactive (synergistic) effects for OTA + CIT. Using a porcine renal cell line (LLC-PK1) and the MTT reduction test as a cytotoxicity end point, PAT, OTA, OTB and CIT were tested individually and in combination. The toxicity ranking for single toxin experiments was PAT > OTA ≥ OTB > CIT, with CIT yielding EC_{50} values of >400 µM. Potency orders for combinations was CIT + OTA > OTA + OTB > PAT + OTA and CIT + OTB > PAT + OTB > OTA + OTB, with CIT being more interactive than PAT with the ochratoxins, OTA and OTB (*33*). Knasmüller and coworkers (*34*) also examined OTA, OTB, and CIT for genotoxicity in human liver ($HepG_2$) cells and pointed out that the combined effects of these mycotoxins in food may have an impact on the overall cancer hazard to humans.

In vivo, simultaneous presence of CIT and OTA modified the toxicokinetics of both toxins. CIT favored the excretion of the toxins, resulting in a decrease of CIT and OTA storage in liver and kidney. Nevertheless, the decrease was not similar in all tissues. This could be explained by competition between the toxins for the transporters of organic anion (OAT). These transporters are expressed in renal cells (*35*), and can transport OTA (*36*) and CIT (*37*). Jung *et al.* (*38*) demonstrated that CIT competitively inhibited absorption of OTA. MRP-2 (multi-drug protein-2) is another transporter involved not only in the excretion of OTA into the lumen of proximal tubules (*39, 40*) but also in hepatobiliary elimination of OTA-conjugates (*41, 42*). In addition, H^+-dipeptide co-transporter (*43 ,44*) mediates OTA reabsorption. This latter transporter is regulated by cylo-oxygenase 2 (COX-2) (*45*). CIT induces COX-2 expression (*30*). Interestingly, there was a synergistic effect on the transport of either tetraethylammonium (TEA) or paraminohippurate (PAH) ions when renal cortical cubes prepared

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from kidneys of young adult Hormel-Hanford miniature swine were treated with OTA and CIT (*46*).

In our study, genotoxicity was measured using ^{32}P-postlabelling for DNA adduct detection. In both cases specific DNA adduct patterns for each mycotoxin were observed. For OTA (1 µM), adduct levels were ~ 64/10^9 nucleotides, while levels for 10 µM CIT were ~ 88/10^9 nucleotides; no adducts for CIT were detected at 1 µM. Interestingly, the combination of OTA (10 µM) + CIT (50 µM) generated mainly OTA-derived adduct spots (~ 50/10^9 nucleotides) with the levels of CIT-specific adducts being 2/10^9 nucleotides. These studies showed that CIT was genotoxic at doses higher than OTA and that the mixture of the mycotoxins favored OTA-mediated DNA adduction. DNA adduct patterns of rat kidney after 3-weeks feeding showed similar adduct spots to those observed in cell culture. Levels of the main OTA-DNA adduct, ascribed to the C-C8 GMP-OTA adduct, were increased by the presence of CIT. This adduct was found in kidney of humans contaminated by OTA (*47, 48*). Genotoxic effects of OTA are related to its biotransformation (for a review see Pfohl-Leszkowicz and Manderville (*2*). Induction of COX-2 also increases the formation of this adduct (*47*). Induction of COX-2 by CIT (*30*) thus favored the biotransformation of OTA into a genotoxic compound. Moreover, the quinone methide structure of CIT could easily explain generation of a DNA adduct (*2, 49*) which may be capable of oxidising OTA into the phenoxyl radical to promote C-C8 adduct formation. This would represent a non-enzymatic pathway for OTA bioactivation that could play a key role in the synergistic effects observed for OTA + CIT.

Altogether our data pinpoint the importance of taking co-contamination into account in the risk assessment of mycotoxins.

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Chapter 4

Carcinogenesis by the Fumonisins: Mechanisms, Risk Analyses, and Implications

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The fumonisin B (FB) mycotoxins are natural contaminants of corn and cause a variety of diseases in animals. Long- and short-term studies on the carcinogenic potency of FB_1 in rats provided information that a cytotoxic/proliferative response is required and a no-effect threshold exists for cancer induction. Oxidative damage and the disruption of lipid biosynthesis with the subsequent alteration of arachidonic acid and ceramide levels are key determinants for the altered growth properties of initiated cells induced by the fumonisins in the liver. Dietary modulation of the FB_1-induced altered growth responses could play an important role in developing chemopreventive strategies. An integrated approach whereby various aspects regarding the diverse toxicological effects, level of exposure in humans and the relevant mechanisms involved need to be considered in establishing realistic risk assessment parameters for the fumonisins in humans. The socioeconomic and health status of population groups in developing countries, utilizing corn as a major dietary staple, should be weighed against economic realities and international trade.

In Food Contaminants; Siantar, D., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2008.

Introduction

The fumonisin B mycotoxins were characterized as cancer promoters utilizing a short-term cancer initiating promoting model in rat liver (*1*). Studies in various animal species showed that fumonisin B_1 (FB_1), the major fumonisin produced by *Fusarium verticillioides* (previously known as *F. moniliforme*) is responsible for most of the toxicological effects of corn culture material. These include equine leukoencephalomalacia in horses (*2*), porcine pulmonary edema (*3*), hepato- and nephrotoxic effects in rats and mice (*4*, *5*) and neural tube defects in mice (*6*).

The fumonisins have been implicated in several human diseases due to their natural contamination of human foodstuffs. Associations with esophageal cancer (*7*), liver cancer (*8*), and neural tube defects (*9*) have been reported. However, a causative role of the fumonisins in any of these human diseases still needs to be elucidated. These investigations are hampered by the lack of a sensitive and specific biomarker of exposure in humans. A typical example is changes in sphingolipid metabolism that is disrupted by the fumonisins resulting in an alteration in the sphingosine to sphinganine ratio (So/Sa), which has been utilized as a sensitive marker of exposure in different animal species (*10*). Studies in humans, however, yield equivocal findings (*11-13*), although the general consensus is that changes in the So/Sa ratio lack sensitivity to monitor human exposure. In order to develop relevant risk assessment parameters, studies into the mechanisms of the toxicological effects are still in progress and could provide unique opportunities to develop specific and sensitive biomarkers of exposure in humans.

Carcinogenicity of the Fumonisins

First reports regarding the carcinogenicity of the fumonisins stem from studies utilizing naturally contaminated corn with *Fusarium verticillioides* as well as corn culture material of the fungus in rats (*14*, *15*). Studies on fusarin C, the major mutagenic compound produced by the fungus (*16*), failed to explain the carcinogenicity of the fungus (*17*), leading to the purification and characterization of the fumonisins as potent cancer promoters in rat liver (1). Despite the fact that a recent study implied the presence of genotoxic constituents in corn cultures of *F verticillioides* (*18*), FB_1 satisfies all the criteria for being the major carcinogenic principle produced by the fungus which can effect both cancer initiating and promoting phases of liver carcinogenesis (*19*, *20*). Although the cancer initiating properties of the apparently non-genotoxic fumonisins were not considered as an inherent property during the risk evaluation, recent studies showed that FB_1 induces a clastogenic effect in different cell culture and subcellular nuclear fractions (*21-24*). Studies in rat liver showed that a threshold for cancer initiation exists depending on the time and dose of exposure (*25*). It would appear that FB_1 mimics the cancer initiating properties of genotoxins such as aflatoxin B_1 (AFB_1), as the initiated cell populations can be promoted into focal proliferative lesions by different cancer promoting regimen such as 2-acetylaminofluorene/partial hepatectomy (*25*) as well as phenobarbital (unpublished data). The weak cancer initiating potency of FB_1 could be related to the induction of apoptosis known to occur in the liver and kidneys of rats exposed to the mycotoxin

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(*26*). A specific role for an increased oxidative damage that could adversely affect the cellular genome and hence have an effect on cancer induction has also been proposed, as initiation by FB_1 seems to be associated with hepatotoxicity (*24*, *27*). Evidence of oxidative damage as a possible mechanism for FB_1-induced DNA damage is emerging fast as recent studies showed that high levels of dietary iron (*28*) and South African herbal teas (*29*) significantly enhanced and reduced, respectively, the carcinogenic potency of FB_1 in rat liver. Dietary constituents, therefore, could modulate the carcinogenic potency of FB_1 by affecting critical cellular events that either increase or decrease the susceptibility of a cell to undergo genetic transformation. The different diets utilized in the carcinogenicity studies of FB_1 showed that a high protein diet could predispose the kidney to the carcinogenic effects of FB_1 in male Fischer rats whilst a low protein diet exhibited a protective effect (*30*). Diets low in protein, vitamins and minerals, on the other hand, could have played an enhancing role in the induction of hepatotoxic and -carcinogenic effects. Synergistic interactions with other mycotoxins such as AFB_1 have been reported in rats (*31*) and rainbow trout (*32*). This implies that dietary approaches to reduce the toxic and economic impact of the fumonisins and other mycotoxins could be a viable option in the future as was suggested recently (*33*).

The role of apoptotic cell death and the resultant regeneration in the kidney has been proposed as a driving force for the development of kidney tumors by FB_1 (*26*, *34*). In the liver, however, both apoptotic and necrotic cell death are induced which complicates a similar type of mechanism. When fed to rats, FB_1 reduced the relative liver and kidney weights suggesting that the net rate of cell proliferation is reduced, creating a negative pressure on the organ homeostasis. The latter was proposed to be the main mechanism for the selection of initiated cells, resistant to the toxic and/or apoptotic effects of FB_1. A similar hypothesis was developed for the kidneys of rats where genetically altered initiated cells are resistant to the apoptotic effects of FB_1 caused by the disruption of sphingolipid metabolism (*35*). In general it would appear that the genesis of a FB_1-associated resistant phenotype in an altered cell population in the liver or kidneys forms the basis for explaining their selective progression into neoplasia.

Proposed Mechanisms of Cancer Promotion

The fumonisins were characterized as active cancer promoting principles utilizing a liver cancer initiating-promoting model (*1*). Two different mechanisms have been proposed for the selective stimulation or enhancement of the growth of altered lesions in the liver and/or kidneys by FB_1. The first hypothesis is centered on the disruption of sphingolipid metabolism where the accumulation of sphingoid bases or the depletion of complex sphingolipids results in the induction of apoptosis leading to the stimulation of compensatory regenerative cell proliferation to maintain cellular homeostasis (*26*, *34*, *35*). This continued level of increased compensatory cell proliferation is suggested to be a key driving force in the genesis of early altered proliferative lesions due to an increased susceptibility of proliferating cells to undergo genetic alterations that eventually result in the development of neoplasia. The second hypothesis is based on

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the growth selection of altered cells, induced during cancer initiation, on the basis of selective inhibition of normal cell growth by FB_1 whilst the altered cells escape these growth inhibitory signals (*36*). Both these hypotheses focus on an increase in cell proliferation, through either compensatory or differential growth mechanisms that eventually result in the abnormal growth of an altered cell population leading to the development of cancer. These different approaches to cancer promotion in the liver were originally developed by Farber (*37*), Dragan and Pitot (*38*) and Schulte-Hermann *et al.* (*39*). The current models for cancer promotion by FB_1 integrate some of the basic biochemical mechanisms induced by FB_1 in the liver and kidneys to provide alternative views regarding the selective outgrowth of resistant initiated cells (*40*).

Alterations in Lipid Metabolism

The disruption of lipid metabolism has been implicated in the toxicological and carcinogenic effects of the fumonisins (*41-43*). In the liver of rats the main emphasis was on the disruption of cholesterol, phospholipid and fatty acid metabolism, while the disruption of sphingolipid metabolism has been proposed to be a major mechanism in the development of liver and kidney cancer (*26*, *35*). In both cases a balance between apoptosis and cell proliferation in selected cell populations forms the basis of cancer promotion and the interaction between the different pathways has been proposed (*36*). Changes in the sphingolipid pathway in the liver and kidneys include (*35*, *44*):

- Inhibition of ceramide synthase.
- Accumulation of sphinganine and sphingosine.
- Depletion of ceramide and other complex sphingolipids.
- Accumulation of sphingolipid -1-phosphates.

Alterations in lipid metabolism in rat liver include (*41 – 43*, *45*):

- Increase in the level of phosphatidylethanolamine (PE).
- Increase in cholesterol.
- Inhibition of the delta -6 desaturase.
- Depletion of polyunsaturated fatty acids (PUFA) in phosphatidylcholine (PC).
- Increase in the PE/PC C20:4n-6 ratio.

The changes in the phospholipids and fatty acid parameters closely mimic alterations known to prevail in hepatocyte nodules that are early proliferative lesions induced by the fumonisins and many other hepatocarcinogens (*46*). These lesions showed a reduced level of oxidative damage (*47*) mainly due to an increased level of reduced glutathione and low levels of PUFA. The reduced oxidative status has been suggested to be one of the major growth determinants in neoplastic lesions as oxidative damage is an important signal for apoptotic cell death. As fumonisins are known to

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induce oxidative damage in the liver, resistance against excessive oxidative damage in hepatic lesions could be a major mechanism for their selective outgrowth, whilst the proliferation of normal cells is inhibited. In this regard primary hepatocytes were more susceptible to the cytotoxic effects of FB_1 than Chang liver cells (*48*). The low level of long chained PUFA in Chang liver cells was suggested to be the main determinant for the differential cytotoxicity as it renders the cell more resistant to FB_1-induced oxidative damage. Another important alteration effected by FB_1 is the increased level of C20:4n-6 in the PE phospholipid, resulting in an increase in the C20:4n-6 PC/PC ratio in different subcellular liver fractions (*45*). Changes to the balance of C20:4n-6 in cellular membranes could be important in the regulation of cell proliferation and/or apoptosis. An interactive role between C20:4ω6 and ceramide in the mitochondria was suggested to regulate the balance between proliferation and apoptosis in altered initiated hepatocytes, resulting in their selective outgrowth during cancer promotion. Although FB_1 disrupts ceramide syntase to the same extent in the nodules and surrounding liver tissue, the accumulation of sphingosine in nodules was proposed to also regulate nodular growth via the formation of sphingosine-1-phosphate (*49*).

The disruption of these upstream events, including sphingolipid, phospholipids and fatty acids in the liver, has been associated with the stabilization of cyclin D_1 via the activation of Akt and the inhibition of GSK-3b activity in preneoplastic and neoplastic lesions (*50*). The subsequent elevated CDk4 activity and increased phosphorylation of retinoblastoma (Rb) protein in early as well as neoplastic lesions may be important role players in the hepatocarcinogenic effects of the fumonisins. With respect to cancer promotion, the induction of TGF-β1 and *c-myc* in the liver was suggested to be involved in the proliferation in TGF-β1 resistant cells (*51*). In addition to the increased expression of TGF-α, *c-myc* could contribute to the modification of cyclin D_1 and the inactivation of Rb. The depletion of growth factors and/or disruption of growth factor signaling pathways and the over-expression of *c-myc* could lead to the induction of apoptosis, which could further assist in the selective outgrowth of resistant initiated hepatocytes. The interaction between the different parameters discussed above seems to be the main differential that will allow altered cells to proliferate and eventually result in the development of liver cancer (Figure 1).

This interactive model provides a challenging approach that could impact on our current understanding of the health risks of cancer promoting substances that naturally occur in major staple diets. Information about their existence in humans exposed to the fumonisins could set new paradigms to determine risk. The question arises whether the aspects regarding the carcinogenic potency of the fumonisins discussed above should be included when establishing risk parameters, such as the Provisional Maximum Tolerable Daily Intake (PMTDI), to safeguard humans from their possible adverse effects. As the mechanism of cancer induction is not known and seems to be associated with a chronic toxicity, the fumonisins are regarded as indirect or non-genotoxic carcinogens. However, it has been suggested that an attempt to develop a regulatory policy for indirect carcinogens solely based on a 2-yr rodent study is ineffective and/or inaccurate (*52*). The use of supplementary biological information should be considered with respect to the fumonisins, as the use of nephrotoxicity as the only measure for determining risk seems inappropriate.

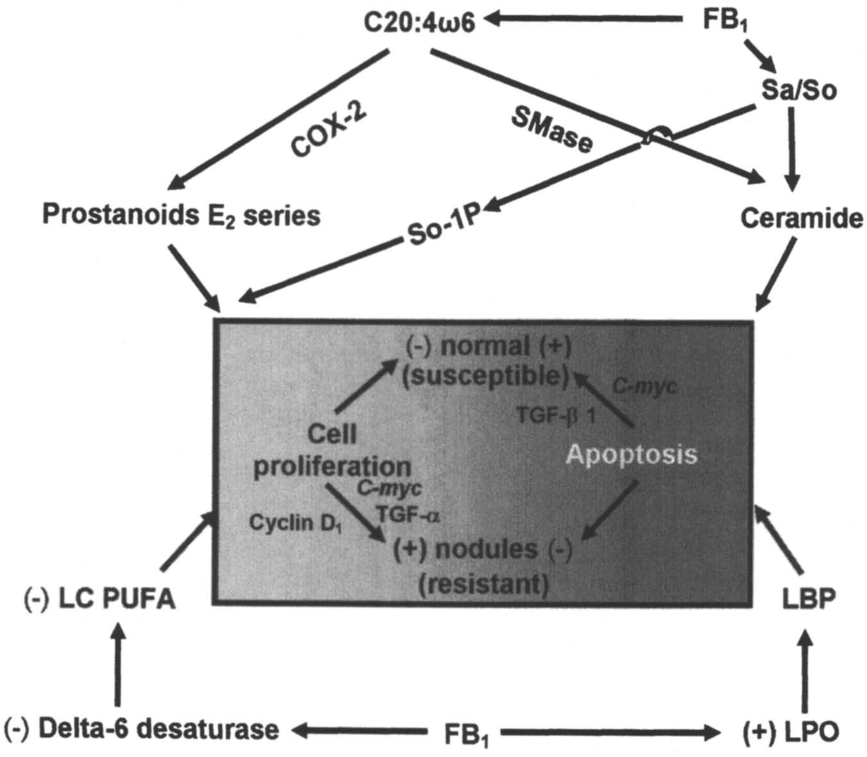
C20:4ω6
FB1
Sa/So
COX-2
SMase
Prostanoids E2 series
So-1P
Ceramide
(-) normal (+)
(susceptible)
C-myc
TGF-β 1
Cell
proliferation
Apoptosis
C-myc
Cyclin D1
TGF-α
(+) nodules (-)
(resistant)
(-) LC PUFA
LBP
(-) Delta-6 desaturase
FB1
(+) LPO

Figure 1. Biphasic effect of FB_1 on the normal vs hepatocyte nodules in the liver of rats. FB_1 increases the level of C20:4ω6 in the liver that selectively stimulates the proliferation of hepatocyte nodules via the induction of prostaglandin E_2 series and regulating apoptosis through ceramide via the sphingomyelinase. FB_1 also inhibits the delta-6 desaturase which results in a low level of LC PUFA and low level of oxidative damage known to stimulate cell proliferation in the hepatocyte nodules. In the normal cells, apoptosis is increased either by the low levels of ceramide or the accumulation of sphingosine or sphinganine, low levels of LC PUFA and the lipid breakdown products as a result of the FB_1-induced oxidative damage. The differential effects of growth regulatory signals such as sphingosine-1-phosphate, C20:4ω6 and ceramide on downstream events such as cyclin D_1, TGFα and β-1, c-myc could selectively stimulate the proliferation of hepatocyte nodules resulting in neoplastic development. LBP=lipid breakdown products; LPO= lipid peroxidation.

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Risk Assessment Parameters of the Fumonisins

The Joint FAO/WHO Expert Committee on Food Additives (JECFA) used the No Observed Effect Level (NOEL) for nephrotoxicity and a safety factor of 100 to provide a PMTDI of 2 μg/FB/day/kg bodyweight (*53*). The numerical size of the safety factor used may differ depending whether a compound is classified as having toxic effects or upon long term exposure could result in the induction of a carcinogenic response. In general larger safety factors are used for carcinogenic compounds depending on the mechanism of cancer induction related to epigenetic (non-genotoxic compounds) or genotoxic mechanisms. When using a safety factor of 1000 for carcinogenicity, the NOEL for hepatocarcinogenicity in male BD IX rats resulted in a Tolerable Daily Intake (TDI) of 0.8 mg FB_1/day/kg body weight (*40*). The NOEL for nephrotoxicity was obtained from a long-term chronic feeding study (2 yr) in male Fischer rats and was equivalent to a dietary level of 5 mg FB_1/kg diet, resulting in a FB_1 intake of 200 μg/kg body weight/day (*34*). A 90 day study in male Fischer rats also provided a similar NOEL for nephrotoxicity (*54*). However, when considering the carcinogenicity of FB_1 the NOEL for nephrotoxicity of 200 μg/kg bw/day is below the no-effect threshold for carcinogenicity (700 μg/kg bw/day) for the induction of adenoma and carcinoma in the kidneys. The NOEL for nephrocarcinogenicity and the hepatocarcinogenicity are very similar and vary between an FB_1 intake of 700 μg to 800 μg/kg bw/day, respectively. A TDI of 0.8 μg FB/kg bw/day, using the NOEL for hepatocarcinogenicity and a safety factor of 1000 has been proposed (*40*) which is very similar to the 0.7 μg/kg bw/day using the NOEL for nephrotoxicity with a similar safety factor. The latter approach resulted in a PMTDI that is in the order of 3 times lower than the PMTDI using the NOEL for nephrotoxicity. At present it is not known whether a PMTDI, based purely on the nephrotoxicity whilst ignoring the carcinogenic properties, will safeguard humans from the adverse effects of the fumonisins.

When comparing the risk parameters between different mycotoxins, the PMTDI for deoxynivalenol (DON) is 1 μg/day/kg bw, using a safety factor approach of 100 based on toxicity (*55*). The level is lower than that calculated for fumonisins which exhibit carcinogenic properties and could have a more adverse impact during chronic low levels of exposure. This becomes apparent when considering the Provisional Tolerable Weekly Intake (PTWI) of ochratoxin A (OA) which is 0.1 μg/kg bw/week which was obtained using a safety factor of 1500 and the NOEL for nephrocarcinogenicity in male rats (*56*). When considering the carcinogenic risk to humans, the International Agency for Research on Cancer (IARC) has evaluated DON as not classifiable as to its carcinogenicity and OA as a possible carcinogenic (group 2B) to humans (*57*, *58*). Some discrepancy therefore exists when considering the PMTDI of fumonisins exhibiting both hepato- and nephrocarcinogenic effects but, like OA, classified a group 2B carcinogen (*58*). At present two tolerable limits exist i.e., a lower limit 0.7/0.8 μg/kg bw/day (lower risk) that focuses on the carcinogenic properties of the fumonisins and a lower limit of 2 μg/kg/day (higher risk) that was based on the nephrotoxic effects. In developed countries exporting large quantities of corn, a lower tolerable limit (0.8 μg/kg/day) could severely affect the corn industry whereas in developing countries with a high corn intake profile and high level of contamination, even the lower tolerable limit will pose a health risk. In this regard it was suggested that a maximum tolerance level (MTL) of 100 to 200 μg fumonisins/kg

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corn should safeguard human health in South Africa when considering the high corn intake profiles in certain rural and urban environments (*59*). In developed countries higher MTL's would be acceptable, e.g., 1 mg fumonisins/kg corn in Switzerland (*60*) due to the low intake profiles in the European Community. Similar situations prevail in France and the USA where levels of 3 and 2 to 4 mg fumonisins/kg whole corn, respectively, have been proposed (*61*, *62*). However, certain population groups in developed countries consuming higher quantities of corn or corn products could be at risk, such as people with celiac disease that consume 162 g/person/day (*63*). A lower PMTDI based on carcinogenicity of the fumonisins could safeguard certain high intake corn consumers worldwide but could also have negative economic impact on international trade affecting the entire corn industry (*64*). As suggested above, different scenarios regarding risk assessment of the fumonisins therefore exist which could either underestimate the risk posed to human health or overemphasize the risk that would negatively impact on international trade and the corn industry in large exporting countries. The current PMTDI of the fumonisins has to be considered against the background of different determinants including health, economy, technology, and politics (Figure 2). The question exists whether the PMTDI for fumonisins addresses the health risk to human populations in developing countries or is in fact driven by economic considerations, protecting the corn industry and international trade agreements of developed countries.

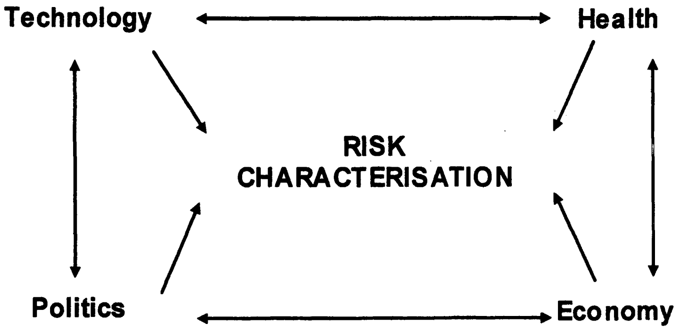

Figure 2. Interactive role of different parameters determining the risk and regulation of food contaminants such as the fumonisins. A balanced approach is required to ensure maximum protection (health PMTDI) of human populations worldwide while conserving the economic viability of corn production and international trade (economic PMTDI).

Fumonisins as Food Contaminants in Developing Countries

Differences exist in establishing and implementing risk assessment parameters from a global perspective versus remote microenvironments in developing countries where corn is used as a sole dietary staple. The health issues associated with these micro-environments are largely ignored by global trade between industrialized countries despite the potential negative impact on human health due to the exposure to

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foodborne toxins and carcinogens. In order to evaluate the risk of fumonisin contamination of corn, the production and consumption profiles of corn in different countries need to be considered. According to the FAO corn production figures, Africa (41.6 million metric tons) is the third largest producer of corn globally following the United States of America (298.2 million metric tons) and China (131.1 million metric tons) (*65*). However, according to the food balance sheets corn consumption profiles, Africa is the largest consumer exceeding 36 million metric tons in 2002 followed by China (20.1 million metric tons) and the USA (3.9 million metric tons). In a recent paper it was argued that corn is replacing sorghum, a staple diet of black people in Africa due to the higher yield (*66*). It is clear from the FAO database on corn production that since 1961 there was a steady increase in corn production in West African countries with a decrease of sorghum and millet production while in southern Africa corn remained the dominant crop compared to millet and sorghum (Figure 3) (*65*).

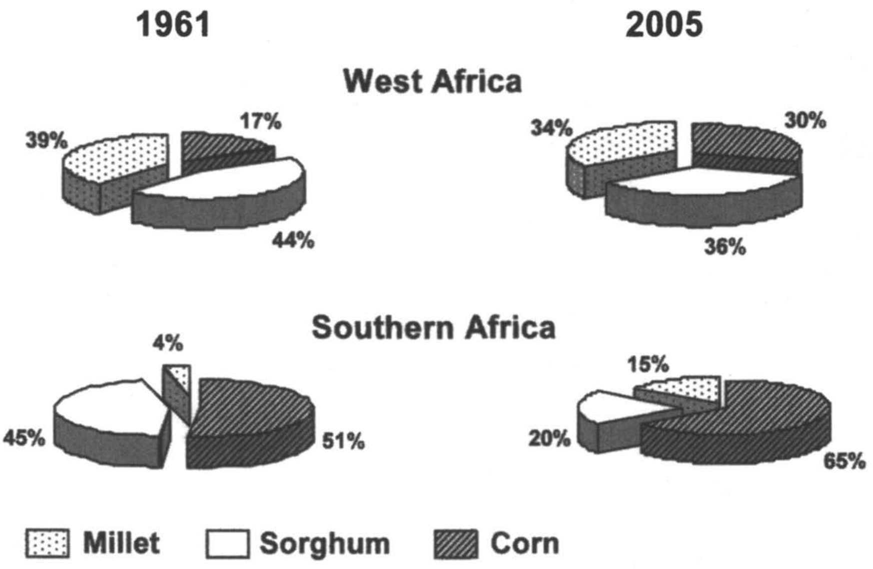

Figure 3. Changes in the millet, sorghum and corn production profiles between 1961 and 2005. Each segment represents the relative % of each crop relative to one another as projected by the FAO.

The dependence on corn as a basic dietary staple in Africa resulted in major imports during droughts typical of the continent. Countries such as the USA, Brazil, and Argentina are large exporters of corn, but apart from the usage for animal feed only 10 % of the harvest is consumed by humans (*65*), mainly as processed foods that significantly reduced the level of FB contamination (*67*). In the African context corn directly enters the human food chain and in many areas it is used as a mono cereal diet for the larger part of the year when the deficiency of micronutrients may exacerbate the susceptibility of consumers to adverse effects of mycotoxins such as the fumonisins.

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Persons who are at the greatest risk with respect to fumonisins in corn are subsistence farmers and children in rural Africa who consume large quantities of mostly contaminated home-grown corn or imported corn contaminated with high levels of fumonisins. Different scenarios therefore exist with respect to the corn production and consumption patterns in different regions and the level of fumonisin contamination. In order to further evaluate the possible risk that fumonisins could pose to humans these parameters should be considered against the current PMTDI when utilizing an interactive approach (Table I).

Fumonisin exposure in rural settings in Africa reaches levels that are far above the PMTDI levels set by JECFA especially when considering the maize consumption patterns and fumonisin contamination levels in different so-called "hotspots" of exposure. In certain of these reports corn intake values approach the 500g/day/person with fumonisin contamination levels exceeding 1 mg FB/kg corn. A recent study in Burkina Faso (*68*) indicated that the mean exposure of people consuming market purchased corn would be 12 to 60 times the PMTDI level of fumonisins. It was suggested that children and infants represents a more vulnerable group as they consumed larger amounts per unit body weight. A study in the Western Highlands of Cameroon showed mean FB levels between 1.74 to 5.8 mg/kg in homegrown corn earmarked for human consumption (*69*). In the Transkei region of South Africa, FB intake profiles showed approximately 3 to 7 and 2 to 4 times the PMTDI levels in children and adults respectively, when consuming home-grown corn (*70*). Studies in other developing countries such as Argentina showed FB intake profiles of up to 1.5 and 5 times the PMTDI in adults and children, respectively (*71*), while in China intake levels in adults varying from 1 to 4 times above the PMTDI have been reported (*72*).

Mean FB contamination levels in white corn cultivated in the commercial maize production areas in South Africa recorded over a period of 6 crop years (1989 to 1994) were 0.28 to 0.78 mg/kg corn (*64*). With an approximate intake of commercial corn of 200 g/day in children in the Eastern Cape Province of South Africa (*73*) at the upper contamination level of 0.78 mg FB/kg, a level of approximately 4 times the PMTDI is calculated. When considering the urban South African consumption patterns amongst adults of 267 g/day (*40*) the intake profile is 1.5 times the PMTDI. Imported corn from the USA during 1992 contained considerably higher FB levels that varied between 2.23 to 2.65 mg FB/kg (*74*). If the imported corn enters the human food chain in South Africa a 10- and 4-fold intake above the PMTDI is calculated for children and adults, respectively, in an urban setting. When considering the higher corn intake of adults at levels up to 500 g per person per day for a rural population, a further 2 times increase above the PMTDI is obtained, a situation that is worsened if home-grown corn is utilized as discussed above. This is of particular importance during dry seasons when large quantities of corn need to be imported, some of which is channeled into the human food chain. As commercial South African corn contains low levels of FB compared to corn supplied on the international markets, the latter could increase the risk due to FB intake in the local population. In addition, impoverished rural communities in Africa depending on corn as a sole dietary staple and with a reduced intake of micronutrients, could be exposed to the adverse health effects by the consumption of corn containing high FB levels. Children appear to be the most vulnerable group of the population and PDI values of 2-3 fold above those

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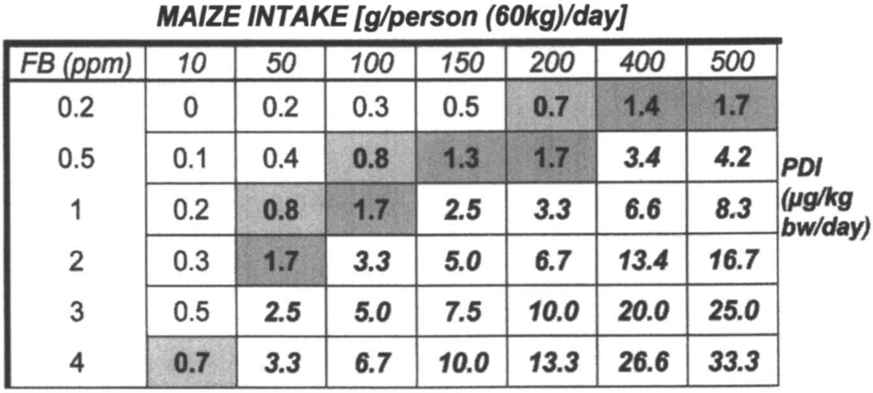
Table I. The probable daily intake of fumonisins, as a function of corn intake profiles and FB contamination level, compared to the PMTDI level related to toxicity and carcinogenicity. The light shaded areas reflect the PMTDI for carcinogenicity (*40*) and the dark shaded areas that for nephrotoxicity (*53*).



MAIZE INTAKE [g/person (60kg)/day]

FB (ppm)	10	50	100	150	200	400	500
0.2	0	0.2	0.3	0.5	0.7	1.4	1.7
0.5	0.1	0.4	0.8	1.3	1.7	3.4	4.2
1	0.2	0.8	1.7	2.5	3.3	6.6	8.3
2	0.3	1.7	3.3	5.0	6.7	13.4	16.7
3	0.5	2.5	5.0	7.5	10.0	20.0	25.0
4	0.7	3.3	6.7	10.0	13.3	26.6	33.3

PDI (µg/kg bw/day)

PMTDI = 2 µg/kg bw/day (nephrotoxicity)
PMTDI = 0.7/0.8 µg/kg bw/day (carcinogenicity)

calculated for adults are obtained. Children are considered to be more susceptible to the adverse effects of mycotoxins as they general have a higher intake on a per kg body weight basis (*75*). Aspects of under-nutrition and the HIV AIDS epidemic in sub-Saharan Africa can only further exacerbate the risk to fumonisin exposure.

The established risk assessment parameters for the fumonisins seem to be limited and failed to safeguard humans residing in developing countries such as Africa with high corn intake profiles and more susceptible to the adverse effects of these mycotoxins. The MTL of 2 to 4 mg FB/kg milled corn as proposed by the USA (*62*) and recently suggested for South Africa (*64*) could further worsen the situation with respect to the proposed PMTDI value. Major obstacles that prevail and lack of regulation in developing countries are ignored when setting risk assessment parameters whereas FB intake levels of 10 to 20 times above the PMTDI are common. It is not known at present what effects the long-term exposure to fumonisins at these levels would have on the health status of specific populations at risk. The need to perform studies to quantify the exposure patterns as well as determining the nutritional status of the people at risk is self-evident. Proper intervention measures to reduce exposure to match the established risk assessment parameters is therefore of critical importance in developing countries and/or the setting of a new PMTDI level to safeguard human population groups in developing countries is urgently required. A MTL of 0.2 mg FB/kg maize as suggested by Marasas (*59*) appears to be remarkably close to a safe contamination level of maize in a South African setting where adults may consume up to 500 g and more maize per day. In children, however, the situation is even worse with a corn intake of up to 200 g/child/day according to a recent survey in the Eastern Cape Province of South Africa (*73*) resulting in PDI levels that far exceed the PMTDI level proposed by JECFA (*53*).

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Several aspects regarding the regulation of FB in corn are of importance. Setting a MTL for FB could either negatively impact on human health in certain population groups or severely disrupt the corn industry and international trade. The setting of realistic risk assessment parameters in an African context where the health of certain population groups and corn production are not compromised requires urgent attention. Aspects regarding international trade and economic realities have also to be taken into account. The complexities and implications of setting control measures of toxic food contaminants have been debated recently (*76*, *77*). When taking AFB_1 as an example, losses in nut export from Africa will be in the order of $40 million if the EU standard of 4 μg/kg is adopted (*77*). If the guidelines of 2 mg/kg with respect to fumonisin set by the FDA isadopted the total export losses to the USA, China and Argentina would be about $100 million, but if this is reduced to 0.5 or 0.2 mg/kg as suggested by Marasas (59), losses will amount to an excess of $300 million (77).

Since African countries rarely export corn the economic loss due to international trade will be minimal while control measures to safeguard its inhabitants should be the focus. However, if mycotoxin monitoring programmes are not in place in Africa, the implementation of a MTL for exposure at the proposed safety limit for fumonisins will not be possible.

The following aspects regarding the regulation of fumonisin mycotoxins need to be considered in the future:

- Introducing risk assessment parameters should consider that people who are at the greatest risk are subsistence farmers and children in certain developing countries, as they have the highest intakes and also consume the most highly contaminated corn. Food security is a constant problem for populations in developing countries leading to under-nutrition that could increase their susceptibility to the adverse effects of mycotoxins. The synergistic interaction of the fumonisins with other dietary constituents such as aflatoxin contamination, increased levels of dietary iron and diets low in antioxidants could predispose these populations to the adverse effects of fumonisins.
- The carcinogenic potential of the fumonisins needs to be included in determining the risk assessment parameters which will result in a 3x reduction in the PMTDI value (*36*). Such a PMTDI should include aspects regarding its cancer inducting potency and the disruption of growth regulatory signals within a cell that could, upon chronic exposure at low doses, lead to the induction of specific cell populations with the potential to develop into cancer. Such an approach will be in accordance with the carcinogenic evaluation of FB_1 by the International Agency for Research on Cancer as a Group 2B carcinogen (*58*).
- The establishment of a MTL in corn that is either too high due to economic considerations or too low to reduce a possible health risk to humans needs to be debated. The technological feasibility to reduce mycotoxin contamination should be developed to comprise both aspects and to sustain a viable corn industry worldwide. At present the proposed MTL level of 2 to 4 mg FB/kg in dried milled corn products (*62*) could adversely affect human health if entered into the human food chain in developing countries.

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- Effective control of fumonisin contamination of corn or development of chemopreventive measures to reduce its impact would be important ways to reduce FB exposure and the associated risk to human health.

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Chapter 5

Understanding Plant–Fungus Associations as a Key to Mycotoxin Control

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This paper describes the influence of plant–fungus associations on the production of mycotoxins. The formation of a particular mycotoxin in a crop depends on a number of factors, but one of the most important is whether the causal fungus has an association with the plant producing the crop. Ochratoxin A is produced by species that do not appear to ever participate in a plant–fungus association, while the fumonisins, trichothecenes, zearalenone and patulin occur in food crops only as a result of such an association. Aflatoxin is unusual: it is formed both as the result of an interaction between plant and fungus and also in the absence of such an association.

The formation of mycotoxins in foods and feeds results from the growth of common species of fungi. However, particular toxins are produced only by specific fungi, and only a small proportion of known foodborne fungi produce important mycotoxins. The detection in a food or feed of a particular mycotoxigenic fungus, known to produce an important toxin, does not automatically mean that that particular toxin will be formed in appreciable quantities. Growth of each fungus is governed not only by environmental factors but also by the association that fungus may have with a particular crop plant or plants, or whether such an association exists. These associations often hold the key to whether significant levels of mycotoxins will be produced by a particular crop in a particular region. Mycotoxin research to date has mostly focused on detection and quantification of particular mycotoxins and general

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principles of agricultural practice, while largely ignoring questions about the specifics of the growth of mycotoxigenic fungi – and thus which mycotoxins are produced in particular foods and feeds.

It is now well known that particular mycotoxins are likely to be produced in only a small range of crops. This paper will outline some of the reasons why this is so, and equally, why it is a waste of resources to look for mycotoxins in crops where their presence is unlikely.

Association versus Absence of Association

The most important basic consideration controlling mycotoxin production is whether the particular fungus has an association with a particular plant species. Mycotoxin production in a crop sometimes results from association of a fungus with a crop while the crop is growing, before harvest. The association may be as a commensal, i.e., living with the plant but not affecting its growth, or as a pathogen. Such associations are usually quite specific for both fungus and plant. However, many other fungi that produce mycotoxins have no association with a plant, in which case invasion of a crop (and mycotoxin production) occurs postharvest, after the crop is picked or pulled, or during drying, transport and storage. Here the growth of a particular fungus in a food or feed commodity is dependent on external factors, including water activity, temperature, etc., and on whether spores of that fungus inhabit the environment around where the crop is grown or dried.

The distinction between these two types of fungal invasion of crops or foodstuffs is an extremely important one, all too frequently overlooked. The control of toxin formation where an association exists must be based on entirely different strategies from the control of toxins which occur in the absence of an association.

Important Mycotoxins

The most important mycotoxins in world agriculture are aflatoxins, ochratoxin A, fumonisins, the major trichothecenes (nivalenol and deoxynivalenol) and zearalenone (*1*), and in the opinion of some, patulin. These toxins can be divided into two classes. Ochratoxin A is the one major mycotoxin that comes from species that do not appear to ever participate in a plant– fungus association. On the other hand, the fumonisins, trichothecenes, zearalenone, and patulin occur in food commodities *only* as the result of a plant–fungus association. Aflatoxin defies such a simple categorization, as aflatoxin is formed in significant amounts both with and without a specific association with

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a plant. This toxin will be discussed first, as a number of important principles can be introduced.

Aflatoxins

More than 40 years after their discovery, aflatoxins remain the most important mycotoxins in the world. It is well known that they are produced principally by two related fungi, *Aspergillus flavus* and *A. parasiticus*. It is also well known that aflatoxins are of particular importance in three crops, maize, peanuts, and cottonseed. It is less well known that the reason that these three crops are the major sources of aflatoxins is because these fungi have an association with them. This association enables the fungus to grow in the plant and in developing seeds and kernels before harvest, providing a big ecological advantage over fungal competitors. Both *A. flavus* and *A. parasiticus* are able to grow in peanut plants and developing peanuts (*2, 3*), while only *A. flavus* has an interaction with maize and cottonseed. *A. flavus* is a commensal fungus in cottonseed (*4, 5*) and probably also in maize (*6*), but the rate of *A. parasiticus* infection in these crops is much lower. It is not known why this difference in associations exists. It should be emphasized that these fungi are not parasites on these crops, but commensals, i.e., the plant does not react to the presence of the fungus, and the fungus grows in the plant without producing any visible damage. Indeed, mature peanuts containing quite high levels of aflatoxins often show no evidence of *A. flavus* infection other than general discoloration, similar to that seen when any of a number of other fungi infect nuts.

It is often important to be able to distinguish between *A. flavus* and *A. parasiticus*. While *A. flavus* produces only B aflatoxins, *A. parasiticus* produces both B and G toxins; only about 50 % of naturally occurring *A. flavus* isolates produce aflatoxins, while virtually all known isolates of *A. parasiticus* are toxigenic (*7*). While *A. flavus* is of universal occurrence in food crops in the tropical and warm temperate zones of the world, *A. parasiticus* has a much more limited distribution. It occurs in association with peanuts in most growing regions and is also a natural pathogen on the sugar cane mealy bug *Saccharicoccus sacchari* (*8*), so is of common occurrence in sugar cane fields. *A. parasiticus* is uncommon in other natural environments. It is also rare in some large geographic regions, including Southeast Asia (*9*).

In summary, aflatoxins in peanuts are believed to be derived from growth of *A. flavus* and *A. parasiticus* in areas of the world outside Southeast Asia, but principally or exclusively only from *A. flavus* in Thailand, Indonesia, the Philippines and Vietnam, and also perhaps other Southeast countries where this has not been studied. A small contribution from *A. nomius* in Southeast Asia also seems likely (*10*). Aflatoxins in maize and cottonseed are mostly or almost entirely produced by *A. flavus*. So while peanuts usually contain both B and G

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aflatoxins, the other major crops where only *A. flavus* is a commensal contain mostly or exclusively B aflatoxins.

Other crops in which aflatoxins may be found include tree nuts such as Brazil nuts, walnuts, pecans, and pistachios, oil seeds including dried coconut, and small grains such as wheat, barley, and rice. In these crops, no plant–fungus association exists, so *A. flavus* (and *A. parasiticus*) lack any ability to systemically invade seeds or nuts, and must compete with a wide range of other fungi for sites of infection. Tree nut species have well developed, tough shells preventing fungal entry unless damage, mainly due to insects, occurs (*11*). As no specific plant– fungus interaction exists here, many different fungal species can infect the nuts (*12*). However, when by chance *A. flavus* does infect a nut, high aflatoxin levels can be produced, as it has often been observed that the presence of oil in any substrate leads to high aflatoxin production.

Pistachio nuts characteristically split their shells, either on the tree around the time of maturity, or subsequently, during drying. If the shells split too early, before the nut is dry, *A. flavus* is one species that will invade and then aflatoxins will be produced (*12*).

Brazil nuts are borne in pods on tall forest trees in the Amazon Basin in Brazil and Bolivia, and are harvested only after they fall to the ground during the rainy season (*13*). Here infrequent harvesting and damp conditions provide time and opportunity for *A. flavus* to infect the pods and nuts, with resultant aflatoxin formation.

Coconut meats are usually dried on the ground or on racks, again providing opportunity for *A. flavus* infection. The drying of spices produces similar problems. These commodities all come from the tropics and, judging from rates of infection in tropical commodities, *A. flavus* is a ubiquitous fungus in these regions (*14-16*).

Figs possess a cavity at the flower end, essential for pollination. Insects carry fungi and yeasts into this cavity, and sometimes *A. flavus* infection results (*17*).

In addition to the potential infection of all of these food commodities around harvest time, *A. flavus* and *A. parasiticus* are xerophilic fungi, capable of growth down to about 0.80 a_w and at temperatures up to 37 °C or more (*18*). Growth of these fungi will therefore continue on crops stored under unsatisfactory conditions, often with aflatoxin formation. This problem occurs particularly in the humid tropics.

Small grains including wheat, barley, and rice rarely contain excessive levels of aflatoxins. Wheat and barley are protected by effective husk enclosure until dry and apparently by low levels of *A. flavus* in fields, perhaps because these fungi do not grow on these plants at all. Rice has the added protection that it is normally hulled soon after harvest, and this process produces heat. Freshly milled rice is essentially sterile (*15*).

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Prevention and Control of Aflatoxin Formation in Crops

For crops where infection by *A. flavus* and *A. parasiticus* occurs only in the absence of a plant–fungus association, prevention of aflatoxin formation involves good agricultural and manufacturing practice. Rapid and thorough drying and effective maintenance of dry storage are the principal mechanisms (*19*). Implementing this advice may be very difficult in practice, but the principles are clearly understood.

Control relies on techniques that permit sorting of damaged nuts, sampling, then testing for aflatoxin levels. Sorting techniques are well developed for peanuts (*20*) and maize (*21, 22*). Similar techniques are used on whole dried figs (*23, 24*). Sorting techniques have been difficult to develop for tree nuts, but some new technologies are showing promise (*11*), including for pistachios, previously considered too difficult because of their greenish color and natural fluorescence (*25*). Insect control in tree nut orchards can be very useful, and insect resistant cultivars of walnuts show promise (*11*). The cultivation of pistachio varieties where shells open late should help to reduce losses of pistachios due to aflatoxin contamination.

For maize, peanuts and cottonseed, crops where a plant–fungus association exists, the control of aflatoxin formation is much more difficult. Conventional plant breeding techniques, often of value for controlling the growth of pathogenic fungi, have been largely unsuccessful against the fungi which produce aflatoxins. This is undoubtedly because these fungi are not pathogens but commensals, and so do not exhibit classic host–pathogen relationships.

In maize, reduction in insect damage by the use of genetically modified cultivars (Bt corn) appears to show promise (*26-28*), though not all authors agree with that (*29*). However, the approach that has been most successful to date has been the technique of biocontrol.

Biocontrol

The type of biocontrol currently in use against aflatoxin formation is correctly called competitive inhibition. High numbers of spores of non-toxigenic strains of *A. flavus* or *A. parasiticus* are introduced into fields where susceptible crops are being grown, where they compete against the naturally occurring toxigenic strains for invasion sites on seeds, nuts or kernels. Considerable success has been achieved by these means in recent years. Approval for commercial processes using non-toxigenic *A. flavus* strains for control of aflatoxin production in peanuts (*30*) and cottonseed (*31*) has recently been granted in the United States, while an application has been submitted for regulatory approval to use non-toxigenic *A. parasiticus* on peanut crops in Australia (*3*). A temporary exemption from the requirement for a tolerance has recently been issued for use of a non-toxigenic *A. flavus* as a biocontrol agent on pistachios in the USA (*32*).

Crops with no Plant– Fungus Association: Ochratoxin A

The ecology of ochratoxin A formation in foods and feeds is complicated by the fact that ochratoxin A is formed in crops by three different groups of fungi of differing ecology. However, the picture is simplified by the second fact that the fungi concerned do not appear to be associated with particular crops before harvest. This greatly simplifies control, at least from the theoretical or ecological viewpoint. The three groups of fungi and the crops affected will be discussed separately.

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Penicillium verrucosum *and the Formation of Ochratoxin A in Cereals*

Penicillium verrucosum occurs commonly in cereals grown in cool temperate climates, ranging across northern and central Europe, Canada and northern Asia. The occurrence of this species in cool temperate cereal crops has two important consequences: ochratoxin A is present in European, Canadian and Japanese cereal products, especially bread and flour based foods, and in the meat of animals that eat cereals as a major dietary component. Ochratoxin A was detected in Scandinavian pig meats 25 years ago (*33*) and its implications for human and animal health were recognised at the same time. As bread, other cereal products and pig meats are major components of European type diets, the further consequence is that many people from the northern cool temperate zone have shown appreciable concentrations of ochratoxin A in their blood (*34-37*).

It is important to note that *Penicillium verrucosum* occurs only in cool climates, as it has a maximum temperature for growth near 30 °C (*38*). This species is responsible for ochratoxin formation only in cereals in the northern cool temperate zone.

Aspergillus ochraceus *and the Formation of Ochratoxin in Stored Products*

Ochratoxin A was originally described as a metabolite of *Aspergillus ochraceus* from laboratory experiments (*39*). It was subsequently reported from several related *Aspergillus* species (*40, 41*). Recently, two new species, *A. westerdijkiae* and *A. steynii,* were split off from *A. ochraceus*, and reported to be the major sources of ochratoxin A in this group of fungi (*42*). *A. ochraceus* itself rarely produces ochratoxin A, and indeed the original producer of ochratoxin A is now classified as *A. westerdijkiae* (*42*). Virtually all of the literature to date uses the name *A. ochraceus* for all three of these species. For many years considered to be responsible only for the formation of ochratoxin A in cereals stored for a long time (*38*), these species have recently been shown to be a major source of ochratoxin A in green (dried) coffee (*43, 44*). Infection has been shown to occur during drying (*43*), and no association between the fungus and the coffee tree has been demonstrated.

Aspergillus ochraceus and these related species are mesophilic xerophiles. Growth of *A. ochraceus* occurs between 8 and 40 °C, while the other two species do not grow at 37 °C. *A. ochraceus* grows optimally between 24 and 31 °C, with a minimum a_w for growth near 0.80 (*38*), and the other species are likely to have similar growth parameters. These species are therefore not likely to infect crops either in the cool temperate zones or while drying under very hot conditions.

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Aspergillus carbonarius *and the Formation of Ochratoxin A in Grapes*

The possibility that ochratoxin A can be produced by *A. carbonarius* and occasionally by its much more common relative, *A. niger,* was discovered only relatively recently (*45-47*). These species have dark hyphae and spores, so are common in vineyards and fruit orchards, where high temperatures and strong sunlight provide a competitive advantage. However, there appears to be no plant –fungus association, as they are not primary invaders of grapes, growing only as the result of damage by plant pathogenic fungi or skin splitting due to unseasonal rain. Growth of *A. carbonarius* in grapes before harvest causes production of ochratoxin A in grape juice and wines. Ochratoxin A occurs in wines from the warmer growing areas throughout the world, but levels are usually low, as the fermentation process positively stops growth of the fungus, and vinification removes most ochratoxin A from the finished product (*48* ,*49*).

In dried vine fruits, however, the possibility of mechanical damage during harvesting and the prolonged time available for fungal growth during drying increase the probability of ochratoxin A being formed. These fungi grow at relatively high temperatures, are notably a resistant to sunlight, and grow down to a moderately low a_w (*50*).

Control of Ochratoxin Production

None of the fungi producing ochratoxin A are known to occur in crops before harvest, i.e., as systemic invaders or pathogens. Although strictly speaking *A. carbonarius* does occur before harvest, it only infects damaged grapes. In consequence, control of the formation of ochratoxin A relies on good agricultural practice. If coffee beans, grapes or cereals are dried rapidly at the time of harvest, and stored dry, little ochratoxin A will be formed. If grapes for wine production are substantially sound, i.e., free from rotting fruit, and are crushed quickly after harvest, leading rapidly to anaerobic conditions, ochratoxin A can be held to low levels. If grains are stored under safe conditions, formation of ochratoxin A by *A. ochraceus* will also be minimal.

Crops with a Plant–Fungus association: Grains and *Fusarium* Mycotoxins

The first important point is that all of the major *Fusarium* species that produce mycotoxins in grains are associated with particular plant species and infect plants and grain before harvest. The second is that all *Fusarium* species grow only at high water activities, above about 0.9 (*38*), so that toxin production in crops occurs only before harvest or during the early stages of drying. Synthesis of toxins ceases long before the crops are fully dry, and only occurs during storage under catastrophic conditions, such as flooding. Production of *Fusarium* mycotoxins occurs as the result of growth of the fungus in the living plant and seed.

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Fumonisins

One major group of *Fusarium* mycotoxins, the fumonisins, are produced by *F. verticillioides* (previously called *F. moniliforme*) and the closely related species *F. proliferatum*. These species are systemic fungi in maize world wide, being always present in the plants and even in healthy kernels (*51*). This is clearly a very close plant–fungus association. Under favorable growth conditions, *F. verticillioides* acts as a commensal and has no effect on the growth of the plant or development of the grain. However, when drought stress or other unfavorable conditions disturb the balance between fungus and plant, fungal growth in developing cobs becomes more obvious, and fumonisins are produced (*51*). *F. verticillioides* has been reported to suppress the growth of other ear fungi (*52*) and heat treated kernels lacking the fungus germinate but do not thrive (*53*), indicating the fungus has a definite role in plant growth. Fumonisin production in maize is favoured by relatively high temperatures (*54*). Fumonisins are normally found only in maize and sorghum, as *F. verticillioides* and *F. proliferatum* rarely infect other crops.

Trichothecenes

The important trichothecenes are deoxynivalenol and nivalenol, produced by *Fusarium graminearum* (often reported as *Gibberella zeae*, its sexual stage) and *F. culmorum*. *F. graminearum* occurs in maize, and both species in small grains, especially wheat and barley. The plant–fungus association here is very close; indeed these species are rank pathogens, invading plants and grains by causing diseases known as Gibberella ear rot and Fusarium head blight. Epidemics of Gibberella ear rot require the congruence of three factors: airborne or insect-borne spores, present at the correct time before harvest, and appropriate environmental conditions of high moisture and low temperature (*51, 54*). Hence these diseases are prevalent in north temperate climates, especially in wet years, and are much less common in wheat grown in the warmer, drier climates prevailing in the southern USA, South Africa, or Australia.

Zearalenone

Zearalenone is produced by the same fungi that produce deoxynivalenol, and usually at the same time. The ecology of zearalenone production mirrors that of deoxynivalenol, at least in general terms.

Control of Fusarium *Mycotoxins*

As with other preharvest mycotoxins, control of the occurrence of *Fusarium* mycotoxins is not easy. Breeding for resistance to Fusarium kernel rot has not been effective so far, although some improvements in disease expression have been seen (*54*). However, good agricultural practice, the control of insects, development of resistance to other ear diseases, and development of cultivars adapted to drought and temperature tolerance are all important in reducing the risk of fumonisin accumulations in maize. Some progress has been made in breeding cultivars resistant to ear rot (*51, 54*).

As noted earlier, the fact that *Fusarium* species grow only at high a_w, means that once grains are dried, increases in levels of *Fusarium* mycotoxins are unlikely to occur.

Crops with a Plant–Fungus Association: Apples, *Penicillium expansum* and Patulin

The major source of patulin in foods is the growth of *Penicillium expansum* in apples and pear fruit. The fungus has no association with the plant, but is a pathogen on the fruit. It causes rapidly spreading brown rots on the fruit, and so is also a major cause of spoilage. The main entry point for the fungus into the fruit is through wounds during harvesting. The use of damaged, rotting fruit during juice manufacture leads to unacceptable levels of patulin in the juice.

Control starts with careful harvesting, and should be followed by rapid processing or cool storage. Hand culling of damaged or rotting fruit before crushing is effective, but expensive. Many juice factories use high pressure water sprays on rotating fruit to cut out rotting areas as part of the process (*55*).

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Chapter 6

Opportunities for Mycotoxin Reduction in Maize Using Biotechnology

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A variety of environmental stress factors increase susceptibility of corn plants to infection with various fungi that produce mycotoxins. These stress factors include insect damage, heat and drought stress, nitrogen deficiency, and genetic susceptibility. For example, insect feeding injures corn kernels, creating ports of entry for fungi that produce ear rot and mycotoxins. Biotechnology is helping to provide season-long protection of corn plants (Bt corn) against corn borer damage through the introduction of insect control proteins derived from *Bacillus thuringiensis.* Reduction of insect feeding damage in Bt corn has led to lower contamination with

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fumonisin mycotoxins in most locations where it has been tested around the world. Biotechnology is also being used to develop healthier corn plants by making them less susceptible to drought stress, increasing nitrogen utilization, and protecting plants against a wider variety of insect pests that feed on the ears, stalks and roots. In the future, combining these traits will further improve yield and should reduce corn plant susceptibility to environmental stress factors that contribute to mycotoxin contamination in the field.

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Corn (*Zea mays* L.) can be infected with fungi that produce toxic secondary metabolites called mycotoxins. *Fusarium verticillioides* (Sacc.) Nirenberg (synonym = *F. moniliforme* J. Sheld.) and *F. proliferatum* (T. Matsushima) Nirenberg, which produce fumonisin mycotoxins, are the most common fungi that infect corn wherever it is grown. Various environmental factors such as insect damage, heat and drought stress, nitrogen deficiency, and genetic susceptibility predispose corn plants to infection with fungi (*1,2*). Dietary exposure to fumonisins can cause a variety of adverse health effects in farm and laboratory animals (*3*). Epidemiological studies suggest a link between high dietary intake of fumonisins and elevated rates of liver and/or esophageal cancer in certain regions of Africa, China, Italy, and Brazil (*3,4*). There are also epidemiological investigations of possible links between fumonisin exposure in early pregnancy and increased incidence of neural tube defects in newborns (*5,6*). As a consequence, regulatory agencies have recommended limits for fumonisin contamination of corn intended for animal feed and human food use (*3*). The United States Food and Drug Administration (*7*) has provided guidance to food and animal feed industries for maximum levels of fumonisin contamination in corn of 2 to 4 ppm (mg/kg) for various corn fractions going into human food. The European Union is proposing setting limits ranging from 0.2 to 2.0 ppm fumonisins in corn intended for use in various human foods, the lowest level being for corn used in baby food (*8*)

Control of insect pest damage to corn can reduce fungal infection since insect feeding provides ports of entry for fungi. Some insect pests also serve as vectors for fungal infection (*9*). An effective insect pest control strategy has been developed with the introduction of coding sequences for the Cry1Ab protein derived from *B. thuringiensis* into corn plants (event MON 810, YieldGard Cornborer®, trademark of Monsanto Technology LLC) (*10*). The Cry1Ab protein controls lepidopteran insect pests such as the European corn borer (ECB), *Ostrinia nubilalis* Hübner, the most important stalk-boring and ear damaging insect pest of corn in the United States Corn Belt (*11*). The CaMV 35S gene promoter enables constitutive expression of the Cry1Ab protein throughout the growing season, thus providing season-long protection against corn borers. The *B. thuringiensis*-based (Bt) microbial pesticides that contain Cry proteins such as Cry1Ab have been used commercially in agriculture for over 40 years to control larval insect pests (*10, 12*). They have an exemplary safety record because their insecticidal mode of action is highly specific against target lepidopteran insect pests. The Cry 1Ab protein has no activity against non-target organisms such as mammals and birds (*10, 12*).

Munkvold and co-workers were the first to report that corn hybrids protected with the Cry1Ab protein had significantly lower fumonisin mycotoxin levels in corn (*13*). This was most evident in corn plants that expressed Cry1Ab protein constitutively during the growing season. Additional field trials have been conducted in countries that permitted field testing of YieldGard Cornborer hybrids to assess their impact on fumonisin levels under local conditions. Results are presented from field trials in the United States, Italy, France, Germany, Turkey, and Argentina that compared the levels of fumonisins in YieldGard Cornborer varieties with their near-isogenic controls.

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Materials and Methods

United States

For the data presented in Figure 1, and Table II, the materials and methods used are described in a publication (*14*).

Germany

Materials and methods information is summarized in the citation included with the data on Table I.

Turkey

The data are presented in Tables I and II. Field trials with YieldGard Cornborer varieties were carried out in Adana province, in the East Mediterranean Cukurova region, where corn is planted as a second crop after wheat. Damage from corn boring insects pests is most severe in the second corn crop. The hybrids used for the study were DK626 Bt, its near-isogenic control (DK626) and a conventional hybrid traditionally grown in the region. The trials were carried out under conditions of natural insect infestation. Trials in 2000 and 2001 were set up using a randomized split plot design with four replicates.

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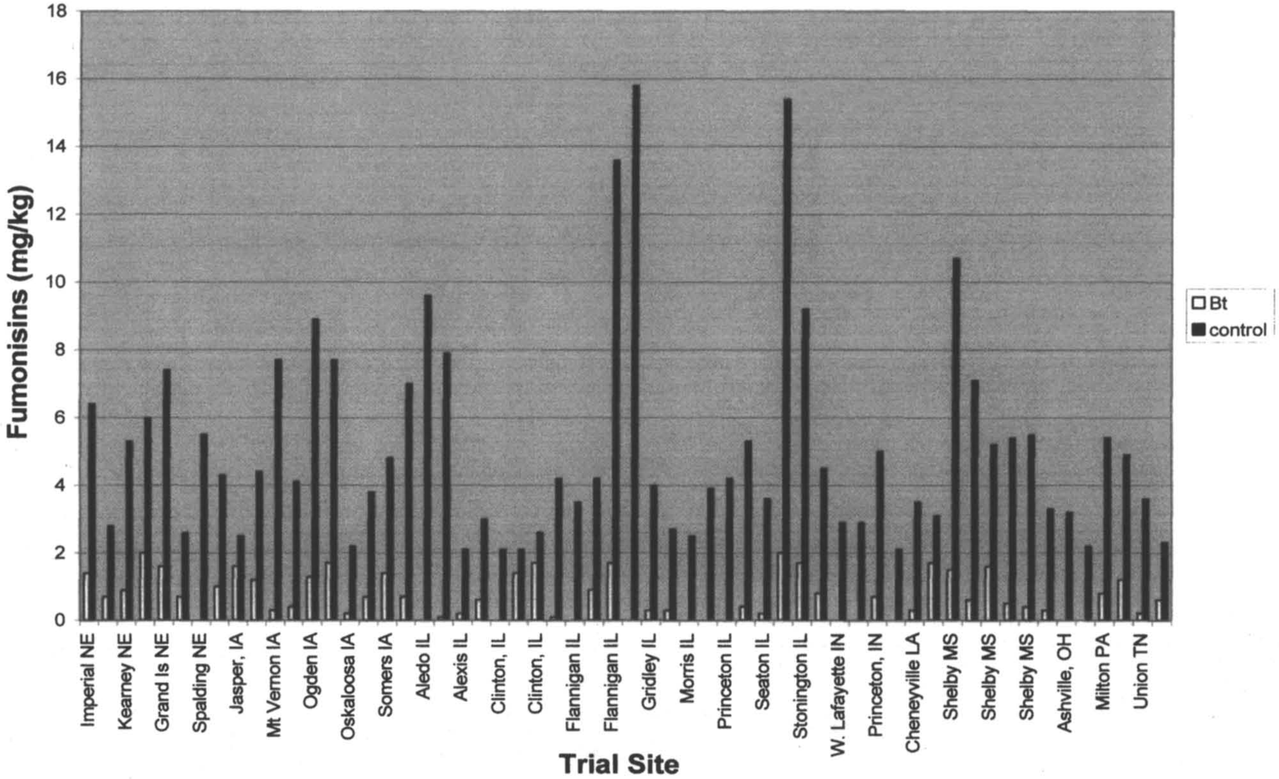

Figure 1. Field trial locations in the US where total fumonisins >2 mg/kg for control hybrids compared to Bt hybrids grown at the same locations. (Reproduced with permission from The Mycotoxin Factbook, Food and Feed Topics; 2006. *Copyright 2006 Wageningen Academic Publishers.)*

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Table I. Lower Fumonisin Levels in YieldGard Cornborer Hybrids Grown in Different Countries

Varieties	*Fumonisin Level (μg /kg)*	*Country*	*Reference*
Novelis Bt (+)	625	Germany (mean across 3 sites)[c]	*19*
Novelis control) (-)	4718	Germany (mean across 3 sites)[c]	*19*
Monumental Bt (+)	nd[a]	Germany (mean across 3 sites)[c]	*19*
Monumental (-)	4665	Germany (mean across 3 sites)[c]	*19*
DK626 Bt (+)	2500	Adana, Turkey (2000)	*20*
DK626 (-)	17,500	Adana, Turkey (2000)	*20*
DK626 Bt (+)	780	Adana, Turkey (2001)	*20*
DK626 (-)	16,750	Adana, Turkey (2001)	*20*
Bt hybrids (+) 3 varieties	2460[b]	Argentina 2000 (mean across 4 sites)	*21*
Near isogenic controls (-)	6290	Argentina 2000 (mean across 4 sites)	*21*
Bt hybrids (+) 4 varieties	560	Argentina 2001 (mean across 4 sites)	*21*
Near isogenic controls (-)	3060	Argentina 2001 (mean across 4 sites)	*21*

[a] Not detected.

[b] Mean value for all varieties across 4 locations.

[c] Manually infested with European corn borer.

(+) Contains Cry1Ab protein.

(-) Does not contain Cry1Ab protein.

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ACS Symposium Series; American Chemical Society: Washington, DC, 2008.

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Table II. % Reduction in Fumonisin Levels Observed in Field Trial Studies with YieldGard Cornborer by Country.

Country	*Year*	*Sites*	*Non-Bt Hybrids Mean Fumonisins (mg/kg)*	*Bt Hybrids Mean Fumonisins (mg/kg)*	*% Reduction in Mean Fumonisins*
USA	2000	49	3.1	1.4	55
USA	2001	125	4.9	2.6	47
Turkey	2001-2002	2	17.5	2.6	85
France	1997-1999	26	1.0	0.03	97
Italy	1999	30	2.9	0.35	88
Argentina	2000	4	6.29	2.46	61
Argentina	2001	4	3.06	0.56	82
Argentina	2000	57	5.03	1.95	61

Each of the four blocks was divided into two sub-blocks. Four sub-blocks received three applications of the insecticide lambda-cyhalotrine at two to three week intervals, starting the third week of July. The other sub-blocks were not treated with insecticide. Test plots consisted of eight corn rows 20 m in length. At harvest, mycotoxin concentrations were compared across treatments. ELISA test kits (quantitative kit for fumonisin, Veratox, Neogen Corp., Lansing, MI, USA) were used to determine fumonisin levels in accordance with the kit instructions.

Italy

The data are presented in Figure 2 and Table II. In 1999, field trials were carried out in northern Italy at 30 different locations with four YieldGard Cornborer varieties of different genotype and their respective near-isogenic controls. The trials were carried out under conditions of natural insect infestation as there is significant ECB infestation in these regions. Approximately 93 samples of corn were randomly collected from one to 10 weeks post-harvest from the various locations and sent to the School of Agriculture, U.C.S.C., Piacenza, Italy, for analysis using published procedures (*15*).

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France

The data are presented in Figure 3 and Table II. During 1997 to 1999, the levels of fumonisin mycotoxins were measured in YieldGard Cornborer varieties and their near-isogenic controls at 25 field trial locations in France. The majority of sites were in the southwest of France where ECB is normally present. The trials were carried out under conditions of natural insect infestation. In non-replicated field trials, the plot size was a minimum of 20 m x 8 rows. In replicated field trials, there were four randomized blocks in two plots (Bt and non-Bt), in plots 12 m by six rows. For non-replicated trials, corn was collected from 20 consecutive plants in two adjacent rows at four locations in the plot. For replicated trials, corn was collected from 20 consecutive plants in two adjacent rows for each replicate. Corn harvested in 1999 trials was analyzed for fumonisins at INRA, Nantes, France according to published methods (*16*). Corn samples harvested in 1998 were analyzed for fumonisins at IEEB (European Institute of Environment at Bordeaux) using established methods (*15*). For corn harvested in 1997, samples were analyzed at AGPM (French Corn Growers Association) using ELISA methods (Diffchamb France, Transia® plate Fumonisins).

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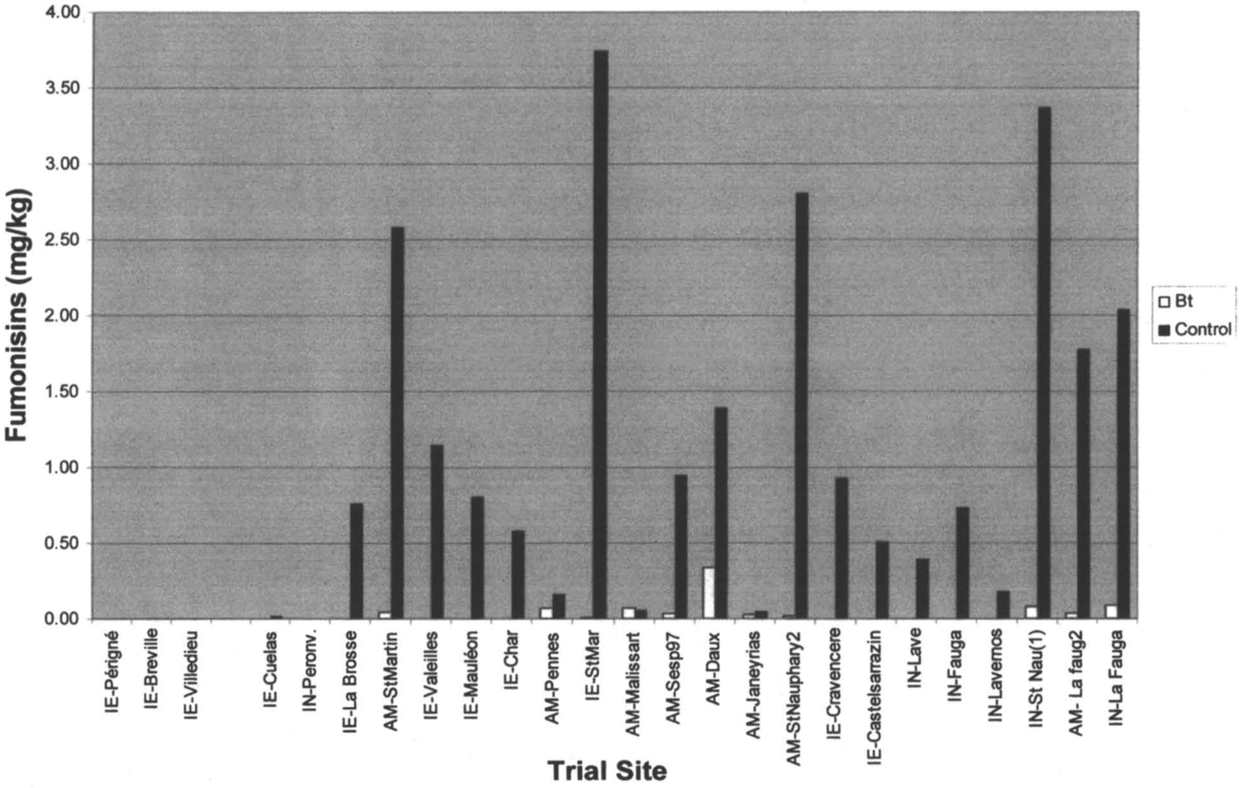

Figure 2. Field trials with YieldGard Cornborer in Italy, 1999. (Reproduced with permission from The Mycotoxin Factbook, Food and Feed Topics; 2006. *Copyright 2006 Wageningen Academic Publishers.)*

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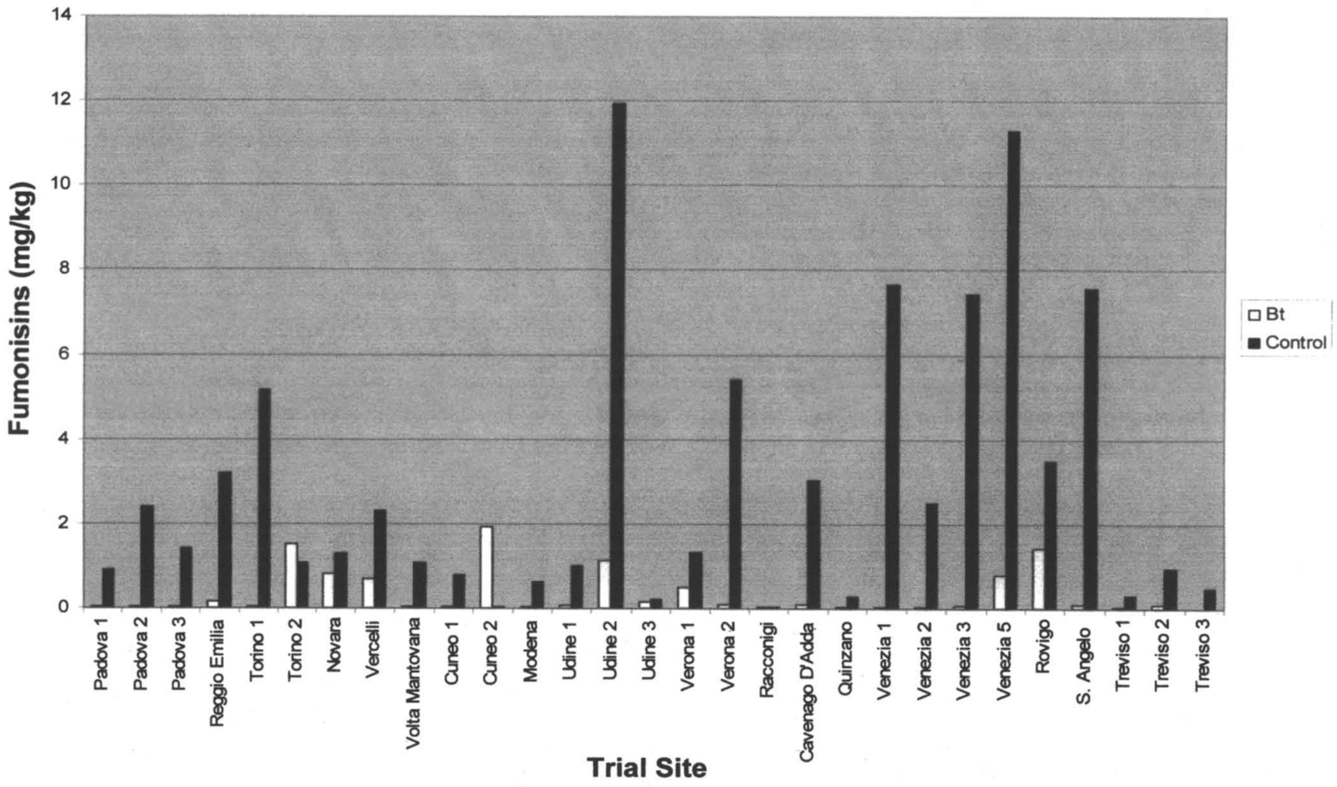

Figure 3. Field trials with YieldGard Cornborer in France, 1997-1999. (Reproduced with permission from The Mycotoxin Factbook, Food and Feed Topics; 2006. *Copyright 2006 Wageningen Academic Publishers.)*

Argentina

For data in Tables I and II, the material and methods information is summarized in the citation given on the table. For data in Figure 4 and Table II, a YieldGard Cornborer hybrid (DK696) and its near-isogenic control (DK696) were grown in 57 different locations in Buenos Aires province in Argentina during 2000. This province is an important corn growing region for Argentina and corn pests such as *Diatraea saccharalis* (corn borer) and *Helicoverpa zea* (ear worm) are frequently found in this region. The field trials were carried out under conditions of natural insect infestation. Corn samples were collected from each of the sites at harvest and submitted for fumonisin analysis at the Laboratorio Tecnológico del Uruguay (LATU) in Montevideo using published methods (*17*).

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Results and Discussion

As shown in Table I and Figures 1-4, many of the field locations had lower fumonisin levels in YieldGard Cornborer hybrids compared to their near isogenic controls. These results confirm that insect feeding is an important contributor to fumonisin contamination of corn under conditions where there is significant corn borer presence. Protecting corn against insect feeding damage can reduce the opportunity for fungi to infect kernels. YieldGard Cornborer hybrids have been reported to have lower fungal contamination based on measurements of ergosterol levels in corn (*16, 18*).

When the levels of fumonisins were averaged across all sites for trials in each country (Table II), the YieldGard Cornborer hybrids had reductions ranging from 47 to 97% compared to their near isogenic controls. At some locations where YieldGard Cornborer was tested, the reduction in fumonisin levels was large enough to make the difference between an unacceptable crop and one that was safe for consumption.

For some published field trial studies, the fumonisin levels in Bt hybrids were compared statistically to controls; the statistical procedures used were summarized in the publications. For the large field trials conducted in the US from 2000 to 2001 (Figure 1 and Table II), the fumonisin levels in Bt hybrids were statistically significantly lower ($p = 0.0007$) than corresponding controls (*14*). In one of the Argentine trials (21) as summarized in Tables I and II, 3 of 4 different Bt hybrids tested had statistically significantly ($p<0.008$) lower fumonisin levels than their corresponding controls. In the field trials in Germany (*19*) as summarized in Table I, fumonisin levels in Bt hybrids were also statistically significantly lower ($p< 0.05$) than controls. In Figures 2-4, the data was not subjected to statistical analysis, but is is readily apparent from inspection of the figures that Bt hybrids had consistently lower fumonisin levels than

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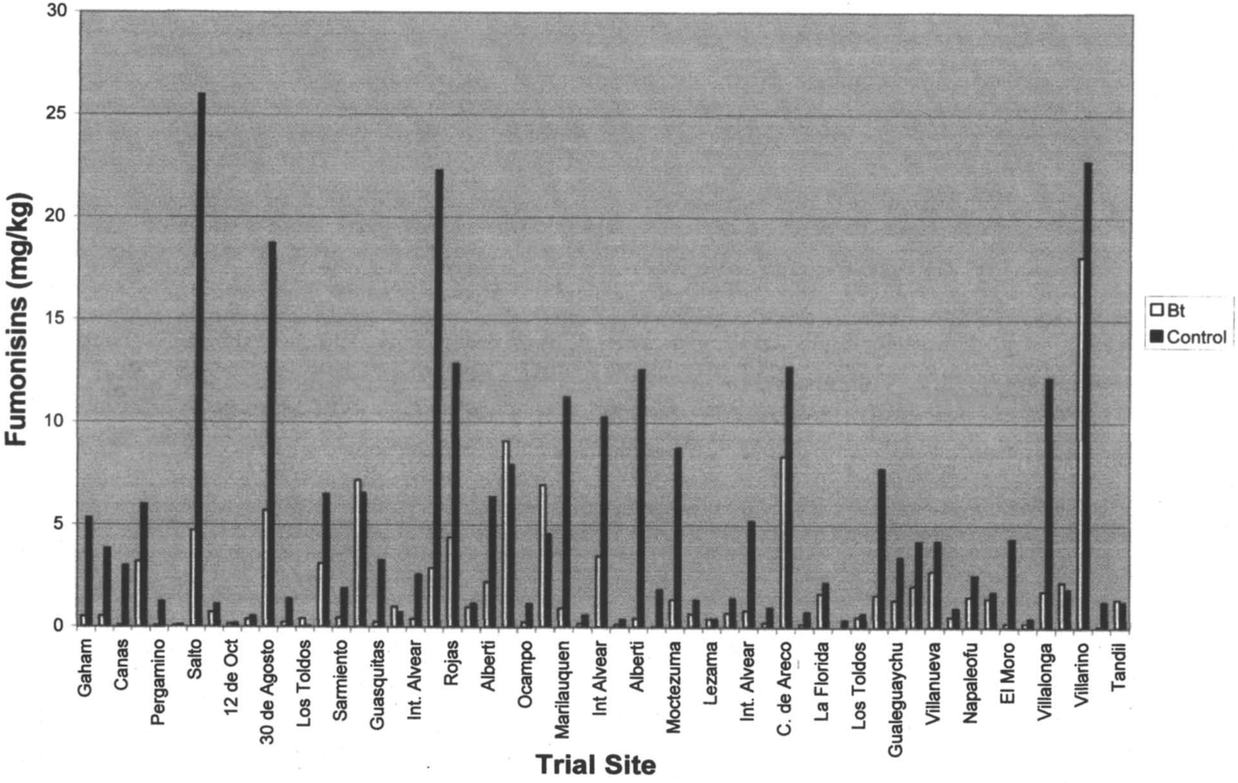

Figure 4. Field trials with YieldGard Cornborer in Argentina, 2000. (Reproduced with permission from The Mycotoxin Factbook, Food and Feed Topics; 2006. *Copyright 2006 Wageningen Academic Publishers.)*

controls. A related published study from France that was referenced earlier (*16*) also reported statistically significantly lower ($p < 0.01$) fumonisin levels in Bt hybrids compared to controls. The weight of evidence suggests that Bt hybrids that constituitively express the Cry1Ab protein throughout the plant during the growing season are less susceptible to corn borer damage and have consistently lower levels of fumonisin contamination in grain.

As one might expect, the environmental conditions in the various field trials conducted at different times in different countries were probably not identical. For most studies, the environmental conditions were not routinely monitored, although in two publications (*19*, *21*), there was discussion of the impact of the environment at specific locations on mycotoxin contamination. A comprehensive study in Argentina designed to assess the impact of environment and insect damage on fumonisin levels in Bt and control hybrids found that environment/weather conditions had a major impact on fumonisin levels. Thus, under some environmental conditions, or where insect pests not well controlled by the Cry1Ab protein predominate, YieldGard Cornborer hybrids may not effectively reduce fumonisin contamination. However, in the many locations where corn borer pests persist, YieldGard Cornborer hybrids can generally effectively reduce fumonisin contamination as illustrated in the field data presented herein.

Significant fumonisin contamination of corn intended for human consumption continues to occur as evidenced by recent reports of its presence in corn meal (mean level 2,200 μg/kg) and corn-based infant food (*22*). Fumonisin and aflatoxin co-contamination of corn has also been reported in some locations (*23*), which may be of concern since fumonisins promote the liver carcinogenic activity of aflatoxin in animal models (*24, 25*). Given the widespread dietary exposure to fumonisins alone or in combination with other mycotoxins, there is an urgent need for more research to assess the potential impacts on human health (*26*). In some areas of the world, the daily dietary exposure to fumonisins from consuming large quantities of contaminated corn considerably exceed the World Health Organization's maximum tolerable daily intake of 2 μg/kg/day fumonisins (*5, 6*).

The economic impact of mycotoxin contamination of food crops has been estimated to range from 0.5 to 1.5 billion dollars/year in the United States alone (*3*). In other world areas, the economic impact of mycotoxin contamination has not been well studied, but is expected to be significant. The economic impact resulting from human health consequences has not been included in the aforementioned estimates. As a consequence of the significant economic impact of mycotoxins, scientists around the world are actively engaged in finding ways to reduce mycotoxin contamination of food crops. Biotechnology is providing tools for researchers to understand the complex dynamics involved in fungal infection and production of mycotoxins. The fungal biosynthetic pathways for mycotoxins such as aflatoxin, fumonisin and deoxynivalenol (DON) are

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complex, involving multiple enzymatic steps. Biotechnology has helped to identify the genes involved in mycotoxin synthesis. Gene-knockout fungal strains are being used to determine key enzymatic steps in mycotoxin production (*27, 28*). The regulation of genes involved in mycotoxin production has been investigated using RNA silencing targeted against mycotoxin biosynthetic pathway-activating transcription factors (*29*). All of these research efforts may help to identify critical control points in mycotoxin production that could provide research leads for future mycotoxin reduction. Some have proposed introducing genes into plants that produce enzymes to degrade mycotoxins (*30*). Others are using biotechnology to help identify natural resistance factors to fungal infection that are present in plants including corn (*31*). Once identified, the coding sequences for these resistance factors may be introduced into susceptible varieties of corn to see if they improve protection against fungal infection.

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Biotechnology is also being used to help develop healthier corn plants, which can withstand environmental stress factors that increase susceptibility to fungal infection in the field. Results were presented for fumonisin reduction in corn plants protected against corn borers. Building on this initial success, the next generation of Bt corn will produce Cry proteins that will provide better protection against a wider variety of insect pests such as fall armyworm and corn earworm, in addition to corn borers (*32*). The overall strategy is to produce corn varieties that are much less susceptible to environmental stress factors in the field and therefore improve yields for farmers. A secondary benefit of these improvements should be reduced susceptibility to fungal infection and mycotoxin contamination.

An example of reduced environmental stress followed the recent introduction of another Bt corn variety protected against corn rootworm feeding (YieldGard Rootworm ®). This variety demonstrated superior performance in 2005 across parts of the midwest United States afflicted with prolonged drought (*33*). The maintenance of a healthy root system afforded better absorption of water and nutrients thus protecting the plant against drought stress compared to conventional corn varieties that were less protected against rootworm damage (*34*). The introduction of herbicide resistant corn varieties can also improve nutrient availability by reducing weed competition that competes for the same nutrients such as nitrogen. Increasing the availability of nitrogen has been reported to reduce aflatoxin contamination in corn (*2*). The combination of root, stalk, and ear protection against insect feeding as well as herbicide tolerance in the same corn plant can reduce plant stress significantly. Ongoing research is identifying genes that reduce drought stress since tolerance to drought may reduce susceptibility to aflatoxin contamination (*2, 35*). Combining these important agronomic traits with those already available will further enhance the ability of improved corn varieties to withstand a broad variety of plant stress factors. We anticipate that these improvements will also lead to further reduced fungal infection and mycotoxin contamination. Thus, biotechnology has opened up many different research opportunities that may lead to significant reduction of mycotoxin contamination of food and feed crops.

Conclusion

Fumonisin contamination occurs wherever corn is grown. Field trial experiments in locations where corn borers are prevalent have consistently found lower fumonisin contamination in the grain of YieldGard Cornborer hybrids. At some locations, the reduction in fumonisin levels was large enough to make the difference between an unacceptable crop and one that was safe for consumption. Biotechnology will provide many tools to help understand the dynamics of environmental stress factors and their impact on fungal interactions with the corn plant resulting in mycotoxin production. This may lead to new intervention strategies to reduce mycotoxin contamination. Developing healthier corn plants to better withstand environmental stress factors will also have secondary benefits of reducing mycotoxin contamination. The application of the many biotechnology tools that are now available should significantly improve crop yield and improve feed and food security, which is of critical economic importance to the world community.

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Chapter 7

A Review on the Use of Mycotoxin Sequestering Agents in Agricultural Livestock Production

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Among the strategies utilized to reduce the toxic effects of mycotoxins on agricultural livestock, the inclusion of sequestering agents to diets is the most commonly practiced. Unfortunately, data on the mode of action of these sequestering agents and *in vivo* experiments published in peer reviewed journals have been limited. In the last 10 years a new wave of interest has led to experiments that have begun to shed light on the effectiveness (or lack thereof) of mycotoxin sequestering agents. The following review summarizes the literature available on the utilization of non-nutritive sequestering agents to minimize the toxicological effects of mycotoxins and to reduce the potential carryover of the toxins into the human food chain. The principal classes of sequestering agents - silicate minerals, activated charcoals, polymers, chlorophyll products, and yeast-derived products - will be discussed.

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Mycotoxins are toxic, biologically diverse, secondary fungal metabolites produced by several fungi, particularly by species of *Aspergillus*, *Fusarium* and *Penicillium*. Molds and mycotoxins occur worldwide and negatively affect animal productivity and human health. Apart from the public health threat, mycotoxins are associated with huge economic losses for both crops and agricultural livestock. It has been estimated that as much as 25% of the world's crops are infected with mycotoxins (*1*). In the United States alone, the U.S. Food and Drug Administration (FDA) estimates the mean simulated livestock cost from the mycotoxins, aflatoxins, fumonisins, and deoxynivalenol, to be in excess of 6 million dollars per year (*2*).

Ever since the first report of a mycotoxicosis in the early 1960s researchers all over the world have been meticulously researching ways to eliminate or minimize the effects of these inevitable contaminants. Due to the wide range of mycotoxins that can contaminate animal feeds and their variable chemical compositions, protection against mycotoxicosis is a relatively difficult task.

Among all the available approaches to control mycotoxin contamination the simplest strategy is based on the prevention of the formation of mycotoxins in feeds (*3*). Even with current technologies it is very difficult to predict or to prevent their occurrence either pre-harvest or during storage and feed processing (*4*). Once ingredients become contaminated with mycotoxins, elimination of the contaminated product is the most effective method of avoiding the problems related to their ingestion (*2*). Unfortunately, due to the difficulties in obtaining a representative sample it is quite difficult to accurately determine the level of contamination of a specific feedstuff. The impracticality and cost associated with the complete substitution of these ingredients means that this practice is not performed as frequently as recommended. Therefore, mycotoxins are often present in animal feedstuffs and pose a substantial economic loss to agricultural animal industries. Moreover, mycotoxins represent a public health concern as animals consuming mycotoxin-contaminated diets may leave residues of these toxins in animal-derived food products.

Mycotoxin **decontamination** refers to methods by which the mycotoxins are removed or neutralized from the contaminated feed while mycotoxin **detoxification** refers to methods by which the toxic properties of the mycotoxins are removed. These strategies include physical, chemical and biological methods. Chemical procedures like treatment with acid/base solutions or other chemicals, ammoniation, ozonation, and reaction with food grade additives such as sodium bisulfite have proven effective in degrading and detoxifying aflatoxin contaminated feedstuffs(*2*). Biological methods primarily involving the degradation of the toxin by microorganisms are receiving increasing interest among researchers and have shown positive results (*2*). Physical procedures like sorting, thermal inactivation, irradiating, or extracting contaminated products have been attempted with variable success (*2*).

Any detoxification or decontamination method for mycotoxin-contaminated feedstuffs should fulfill the following prerequisites.

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1. Be effective in removing, destroying, and inactivating the mycotoxin,
2. Not produce toxic or carcinogenic/mutagenic residues in the treated products nor in the food products derived from animals consuming these treated feedstuffs,
3. Not alter the nutritive properties of the feedstuffs or affect feed acceptability,
4. Be economically and technologically feasible so as not to alter significantly the cost of the final product.

Many of the physical, chemical and biological methods found in the literature are effective in reducing, destroying, or inactivating different mycotoxins but rarely do they meet the other equally important requisites. Nutritional approaches like supplementation of nutrients or additives with protective properties against toxicity, and the addition of non-nutritive sequestrants capable of reducing mycotoxin bioavailability can in fact decontaminate or detoxify contaminated feedstuffs while meeting the other four criteria (*5*). The utilization of nutrients with antioxidant properties to reduce the oxidative effects of mycotoxins has been recently reviewed by Surai *et al.* (*6*). Dietary supplementation with nonnutritive mycotoxin-sequestering agents is by far the most practical and most widely studied method for reducing the effects of mycotoxin exposure. An effective sequestering agent is one that prevents or limits toxin absorption from the gastrointestinal tract of the animal. Ideally, a sequestering agent should be effective against a specific mycotoxin or mycotoxins with similar structure. However, since feedstuffs are commonly contaminated with more than one mycotoxin the utilization of more than one toxin specific product may be necessary. To remain practical, mycotoxin-sequestering agents should also fit into the farm's economic plan and should not occupy a large portion of the complete diet. Additionally, mycotoxin-sequestering agents should be free of impurities, off-flavors, and odors.

One of the most complex aspects of evaluating sequestering agents is in properly classifying these products. Care must be taken not to generalize any conclusions for a given adsorbent group, as all products under a specific classification will not always have the same composition or efficacy. For example 'clays' vary in structure and mineral composition while yeasts used in production of 'yeast cell wall products' differ in cell wall composition. In either case the physicochemical properties that determine sequestrant efficacy can vary markedly among products in the same category.

An effective approach to evaluate mycotoxin sequestering agents should be based on the following key points:

1. Potential products should be evaluated initially in multiple *in vitro* tests (aqueous medium, two-step absorption, absorption isotherms, dynamic gastrointestinal models, etc.).

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2. Products showing strong adsorption for specific mycotoxins *in vitro* should be further evaluated for their affinity to other common components in diets (*i.e.*, minerals, vitamins, and amino acids).
3. The best *in vitro* tested products with low affinity to other diet components should be further evaluated *in vivo* with both single mycotoxin and multiple combined mycotoxins experiments.
4. *In vivo* tests should not only measure performance parameters but should compare specific mycotoxin biomarkers.
5. Long term experiments are required to determine that no negative health or nutritional impacts are caused when sequestering agents are used as a preventive in the diet.

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In Vitro Evaluation of Sequestering Agents

A major obstacle in the evaluation of mycotoxin sequestering agents is related to the inadequacy of *in vitro* models in predicting response in the animal. Several researchers have shown that the ability of a compound to sequester mycotoxins *in vitro* does not necessarily correlate with *in vivo* response. Rotter *et al.* (*7*) selected an activated charcoal that showed a high capacity to sequester ochratoxin A at different pH values *in vitro*. This activated charcoal was then fed to chicks consuming diets contaminated with ochratoxin A. The authors concluded that the addition of the charcoal did not reduce ochratoxin A toxicosis. Similarly, Dwyer *et al.* (*8*) tested three mineral clays (an acidic phyllosilicate, a neutral phyllosilicate, and a zeolite), which had previously shown *in vitro* sequestration of cyclopiazonic acid (CPA). The researchers found that none of the three mineral clays were effective in reducing the toxic effects of CPA in broiler chicks when added to the diet.

Experiments with swine and cattle have shown similar results. Piva *et al.* (*9*) found that an activated charcoal that sequestered fumonisin B_1 effectively *in vitro* did not have any protective properties in growing piglets consuming fumonisin B_1 from a culture material. Diaz *et al.* (*10*) fed several sequestering agents (three bentonites, an activated charcoal, and a yeast glucomannan polymer), all of which had previously shown high aflatoxin B_1 sequestration ability *in vitro*, to lactating dairy cows and measured the change in milk aflatoxin M_1 residues before and after dietary inclusion of the different sequestering agents. The authors found that two of the tested sequestering agents, an activated carbon and a calcium bentonite, had little or no effect in reducing the aflatoxin M_1 concentration.

In vitro analysis of mycotoxin sequestration is however a powerful tool for screening potential mycotoxin sequestering agents. If a sequestering agent does not absorb a mycotoxin *in vitro,* it has little or no chance to do so *in vivo*. These laboratory techniques can be very useful in identifying potential dietary sequestering agents and in helping to determine the mechanisms and conditions favorable for sequestration to occur.

The simplest and most widely utilized *in vitro* method measures adsorption of purified toxin preparations in an aqueous medium. In these systems, a known amount of mycotoxin is reacted with a known amount of test compound in an aqueous solution. The difference between the amount of toxin still present in the liquid after separation and the amount of adsorbed toxin is determined. Due to the relative insolubility of mycotoxins, these tests are generally conducted at very low mycotoxin concentrations. Unfortunately, because of their simplicity these methods have been widely used to evaluate sequestering agents and are the basis of most claims for the effectiveness of certain commercial products.

More elaborate *in vitro* systems utilized to evaluate mycotoxin sequestering agents are based on a two-step process. These systems determine the strength of the mycotoxin-sequestrant complex by first measuring the quantity of toxin sequestered, and then measuring the separation of the mycotoxin from the sequestrant after exposure to a second solvent system. Efficacy is evaluated by comparing the initial sequestration ('weak binding') with that after desorption ('strong binding') (*11*). While such tests are useful in demonstrating the potential of a specific sequestering agent, they are not very efficient for comparisons among different types of compounds. Additionally, these tests provide little information on how a specific compound should be utilized in a practical field situation.

Adsorption isotherms have been effectively utilized to evaluate mycotoxin sequestering agents (*12, 13*). The amount of mycotoxin adsorbed per unit of weight is plotted against the concentration of the mycotoxin in solution at a constant temperature and under stable conditions. This system takes into account that sequestering of mycotoxins is a reversible process that can be characterized as a chemical equilibrium. As a result, absorption is a concentration-dependent process influenced by mycotoxin concentration, the amount of sequestrant, and the relative affinity of the compound for the mycotoxin (*11*).

More recently a dynamic, computer-controlled, gastrointestinal model has been utilized to evaluate different sequestering agents (*14*). However, like other *in vitro* methods proper *in vivo* confirmation is still needed before field utilization can be recommended.

It is extremely important that any *in vitro* results be supported by *in vivo* experiments utilizing the species for which the product is intended and with feeds contaminated with levels of mycotoxins that are commonly found in the field.

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In Vivo Evaluation of Sequestering Agents

Activated Charcoal (AC)

Activated charcoal (AC) is an amorphous form of carbon heated in the absence of air and then treated with oxygen to open millions of pores between the carbon atoms. The carbon source is selected from a variety of materials such as the shells of nuts, wood (coal), moss, etc. This highly absorbent powder has been commonly utilized as a medical treatment for severe intoxications since the 19th century (*15*).

Activated charcoal may be an efficacious product to decontaminate foodstuffs containing aflatoxins or other mycotoxins, although it is probably more suited to use as an antidote for severe toxicity. The sequestrant properties of AC are dependent on many factors including pore size, surface area, structure of the mycotoxin, and dose (*5*). Since *in vivo* data on the effectiveness of AC are variable, careful consideration is needed when evaluating AC utilization. The specific surface area of activated charcoals varies from a low of 500 m^2/g for lignin-based AC to more than 3,500 m^2/g for superactivated charcoals (*16*).

Hatch *et al.* (*17*) effectively utilized AC as an antidote for lethal doses of aflatoxin B_1 (AFB_1) in goats. Animals consuming the AC 8 h after ingestion of 3 mg AFB_1/kg BW had no visible liver lesions and had a lower percentage of hepatic damage (3%) compared to animals receiving no antidote (25%). Following this discovery, Dalvi and Ademoyero (*18*) evaluated the ability of AC to prevent functional alterations of the liver in chickens. When chickens were fed a mixture of 6 mg AFB_1/kg BW and 200 mg of AC/kg BW, functional alterations to the liver (quantity of hepatic cytochrome P450, the rate of microsomal metabolism of benzphetamine and the activity of serum glutamic oxalacetic transaminase (SGOT)) were reduced or prevented.

Dalvi and McGowan (*19*) fed broiler chicks 10 ppm aflatoxin for 8 weeks. One group of birds received 0.1% AC in the drinking water, while no AC was added to the drinking water of the control group. Improvement in feed consumption and weight gain was observed in the charcoal-treated birds. Activated charcoal was also able to reduce the effect of AFB_1 on the microsomal cytochrome P450 and the activities of benzphetamine demethylase and SGOT. The authors proposed that AFB_1 sequestered by the AC becomes unavailable for gastrointestinal absorption.

Kubena and colleagues (*20*) had very different results. Using broiler chicks they found that activated charcoal included at 0.5% of the diet was not effective in reducing toxicity of purified AFB_1 or of aflatoxins from naturally contaminated material and that AC may have even enhanced the toxic effect. The authors were not able to determine a mechanism through which the activated charcoal would enhance aflatoxin toxicity, but hypothesized that it

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may be due to chemical alteration of the sites and the rates of sorption and desorption of aflatoxin. Urinary excretion of aflatoxin M_1, an indicator of absorbed aflatoxin, was reduced by adding 0.5% AC to feedstuffs consumed by turkey poults (*21*). In this experiment however, the performance-associated effects produced by aflatoxin were not ameliorated by dietary AC inclusion.

Bonna *et al.* (*22*) fed a diet containing two levels of aflatoxin (low 34 ppb, and high 102 ppb) to mink for a 77 day period. The addition of 1.0% AC to the diet containing the aflatoxins reduced mortality by 50% in the 102 ppb aflatoxin group and prevented mortality in the 34 ppb group. The increased survival time of the mink was noticeable in this experiment. The AC reduced or eliminated the occurrence of histopathological lesions in the liver but could not relieve the mink of the effects of aflatoxicosis as effectively as a hydrated sodium calcium aluminosilicate (HSCAS) at a 0.5% concentration used in the same experiment. The authors suggested using higher concentrations of AC or superactivated charcoals to improve efficacy.

Galvano *et al.* (*23*) conducted an experiment on late lactation dairy cattle to study the potential of activated charcoal and HSCAS to reduce the carryover of aflatoxin from feed to milk. Using 12 Friesian cows, the authors compared two activated charcoals and an HSCAS at 2.0% of the basal diet DM. One of the two activated charcoals significantly reduced transfer of aflatoxin B_1 to aflatoxin M_1 in milk while the second AC was ineffective in reducing this carryover. Diaz *et al.* (*10*) saw no significant reduction in milk aflatoxin M_1 concentrations when AC was added at 0.25% of the diet to late lactation Holstein cows.

Buck and Bratich (*24*) observed a 70% survival rate (7/10) in rats orally dosed with 6 times the LD_{50} for T-2 toxin (25 ppm) when a commercially available superactivated charcoal was administered immediately after toxin exposure. However, two other AC products tested did not protect the rats exposed to the lethal dose of the toxin. Similarly, Poppenga *et al.* (*25*) dosed swine intravenously 3.6 ppm (3 x LD_{50}), and unexpectedly found that AC was an important part of an oral therapy for acute T-2 toxin exposure. They hypothesized that AC could be sequestering T-2 toxin eliminated in the bile thereby reducing re-absorption of the toxin. Additionally, the authors also theorized that AC may aid animals by binding endotoxins produced by intestinal microbes. Such endotoxins may be more easily absorbed during toxicoses due to the damage to the intestinal epithelium commonly seen in acute T-2 toxin toxicity.

Activated charcoal was ineffective when directly mixed in a complete chicken diet contaminated with 4 ppm ochratoxin A (*7*). The authors concluded that the addition of AC was an impractical method of reducing ochratoxin A toxicity in poultry chronically exposed to this toxin. Similarly, Solfrizzo *et al.* (*26*) found that the sphinganine:sphingosine ratio (Sa:So), a powerful biomarker for fumonisin B_1 exposure, was not modified in rats consuming 2% AC plus fumonisin B_1 as compared to the observed change in Sa:So in the fumonisin B_1-

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fed group. More recently, Piva *et al.* (*9*) found no positive effect of AC on feed efficiency, on serum concentrations of cholesterol, free sphinganine, sphingosine-1-phosphate, and sphinganine-1-phosphate or in activities of γ-glutamyltransferase (GGT) and glutamic oxaloacetic transaminase (GOT) in pigs consuming diets containing 30 ppm fumonisin B_1 from culture material. Interestingly, the authors saw that histopathological effects in liver, lung, heart, pancreas, spleen, intestine, and lymph nodes were more severe in animals given fumonisin B_1 plus AC, suggesting that the AC treatment worsened the toxic effects of fumonisin B_1. The authors hypothesized that the activated carbon could also be sequestering important nutrients such as vitamins, minerals, and essential amino acids, all of which play important roles in the overall health and performance of animals.

Activated charcoal is a relatively nonspecific sequestrant with great potential as an antidote for acute exposure to several mycotoxins. However, the great variability in the results of long-term exposure experiments and its potential for also sequestering important nutrients diminish its overall practical effectiveness for routine dietary inclusion.

Cholestyramine

Cholestyramine is an anion exchange acid-binding resin utilized in human medicine for adsorbing bile acids in the gastrointestinal tract and as a therapy to reduce cholesterol. Cholestyramine has been shown *in vitro* to sequester ochratoxin A (*27*) and zearalenone (ZEA) (*16*). Using rats as an experimental model, Madhyastha *et al.* (*28*) found that cholestyramine lowered ochratoxin A concentrations in plasma. Rats consuming 1 ppm ochratoxin A or 3 ppm ochratoxin A plus cholestyramine had lower enzyme activities (GGT and NAG) as well as higher concentrations of the unmetabolized ochratoxin A. The authors hypothesized that cholestyramine reduced ochratoxin A absorption by either reducing concentrations of bile acids, which may be important in ochratoxin A absorption in the upper gastrointestinal tract, or by direct adsorption of the ochratoxin A molecule. Since the chemical structure of ochratoxin A is quite similar to that of bile acids, it is plausible that both mechanisms are involved. Due to the high cost of this product, its practicality is questionable.

Chlorophyllin

Chlorophyllin, a water-soluble derivative of the green plant pigment chlorophyll, has been shown effective in reducing the toxicity associated with aflatoxin. Several researchers have demonstrated the chemoprotective properties of chlorophyllin (*29-31*). Researchers theorized that the antioxidant properties of

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this chlorophyll derivative were responsible for its chemoprotective abilities. More recently researchers identified that a complex between chlorophyllin and aflatoxin was actually the main mechanism responsible for chemoprotection. Kobayashi *et al.* (*32*) reported that chlorophyllin, when mixed with chitosan (a polyglucosamine), establishes an insoluble salt-like material which can form a non-covalent bond with polycyclic compounds like aflatoxin.

Breinholt (*33*) used rainbow trout to further elucidate the mechanism of activity of chlorophyllin. The authors determined that the formation of the complex between chlorophyllin and aflatoxin B_1 was highly effective in reducing the absorption of aflatoxin B_1 by rainbow trout and that *in situ* target organ protection was an insignificant mechanism of chemoprotection. Based on these reports chlorophyllin can be considered effective in reducing aflatoxicosis. Because of the specific mechanism of adsorption chlorophyllin would only be expected to be effective for aflatoxin and other mycotoxins having a polycyclic structure.

Silicate Minerals

The largest and most complex classes of mycotoxin sequestering agents are the silicate minerals. Within this group there are two important subclasses, the phyllosilicate subclass and the tectosilicate subclass. The mineral clays belonging to the phyllosilicate subclass include important sequestrants like the montmorillonite/smectite group, the kaolinite group, and the illite (or clay-mica) group. The tectosilicates include the important and highly studied zeolites.

Montmorillonite/Bentonite

Montmorillonite is the main constituent of bentonite. Based on exchangeable cation composition, bentonites can be classified as calcium, magnesium, potassium, or sodium bentonites. Due to their montmorillonite content, bentonites swell and form thixotropic gels. Montmorillonites and hence bentonites are used as suspension-stabilizing additives in coatings and, as bonding agents for foundry sands and foundry washes. As a result of their ion exchange capabilities, these products have been widely utilized as mycotoxin sequestering agents.

Masimango *et al.* (*34*) demonstrated early on that several bentonites had the ability to bind to aflatoxin B_1 in a buffer solution. Moreover, Dombrink-Kurtzman and Dvorak (*35*) showed that a bentonite was effective in sequestering aflatoxin B_1 in different liquid media such as water, saline solution, serum of pigs, stomach fluid of pigs, and bovine rumen fluid. Winfree and Allred (*36*) and Ramos and Hernandez (*13*) also demonstrated the high capacity

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of bentonites to sequester aflatoxins in several different solutions. These authors and previous authors have hypothesized that bentonites would sequester aflatoxins *in vivo,* thus reducing aflatoxicosis.

In a series of experiments on growing swine, Lindemann *et al.* (*37*) demonstrated that the addition of a sodium bentonite (0.5%) to diets contaminated with 800 ppb aflatoxin B_1 improved average daily feed intake and increased average daily gain. Bentonite supplementation significantly improved concentrations of blood urea, total protein, albumin and activities of aspartate aminotransferase (AST), alkaline phosphatase (ALP), and γ-glutamyl aminotransferase (GGT), all of which had been significantly altered by the mycotoxin. Schell *et al.* (*38, 39*) also saw a positive effect of bentonite on growing pigs consuming aflatoxin-contaminated diets without negatively affecting mineral metabolism. Similarly, addition of a montmorillonite (5 g/kg) to aflatoxin-exposed rats prevented the degenerative changes in hepatic and renal tissue thus offering protection against serum biochemical parameters affected by the toxin (*40*). These authors postulated that the bentonite formed a complex with the toxin that prevented the absorption of the aflatoxin across the intestinal epithelium.

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Zeolites

Zeolites are framework silicates consisting of interlocking tetrahedra of SiO_4 and AlO_4. The aluminosilicate structure is negatively charged and attracts and holds positive cations within the structure. Zeolites have large pores that provide space for large cations such as sodium, potassium, barium, and calcium and even relatively larger molecules such as water, ammonia, carbonate ions, and nitrate ions. In some zeolites, the spaces are interconnected to form long and wide channels of varying sizes, depending on the mineral composition. These channels facilitate the movement of the resident ions and molecules in and out of the structure. Zeolites are characterized by their ability to lose and adsorb water without damage to the crystalline structure. There are about 45 minerals that are recognized as members of the zeolite group, which can be subdivided into silicate zeolites, synthetic zeolites, and phosphate minerals that have a zeolite structure. The complexity of this combined group is extensive, with over 120 structural variations.

Since zeolites were identified as effective *in vitro* sequestrants of aflatoxin B_1 (*35*) several researchers have studied their *in vivo* response in different animal models. Scheideler (*41*) fed a natural zeolite to broilers consuming 2.5 ppm aflatoxin B_1. The zeolite added at 1% of the diet alleviated the growth depression and reduced the increase in liver lipid concentration caused by the aflatoxin. The zeolite did, however, decrease serum phosphorus (P) and chloride (Cl) concentrations independently of aflatoxin exposure. Sova *et al.* (*42*) showed that zeolite inclusion at 5% of the diet significantly reduced heterophilia and lymphopenia in broilers consuming an aflatoxin-contaminated diet. However, the authors noted that the zeolite did not protect the animals against the acute alteration of liver parenchyma caused by aflatoxin B_1.

Harvey *et al.* (*43*) fed growing broiler chickens a diet contaminated with 3.5 ppm total aflatoxins with or without the inclusion of five different commercially available zeolites. Three of five tested zeolites were not effective in alleviating the decrease in body weight gain associated with the aflatoxins. The other two products were clearly effective in alleviating the aflatoxin-associated problems. More recently, Zaghini *et al.* (*44*) fed layers 2.5 ppm aflatoxin B_1 with or without the inclusion of a zeolite. Zeolite (clinoptilolite) at 2% of the diet was effective in reducing liver concentrations of aflatoxin B_1. Results of a study with pregnant rats were not, however, as positive. Clinoptilolite addition resulted in maternal liver lesions more severe than with aflatoxin B_1 alone. The authors suggested that the clinoptilolite may interact with dietary components that modulate aflatoxicosis (*45*).

Recently, Santin *et al.* (*46*) fed broilers a diet contaminated with 2 ppm ochratoxin A with or without the inclusion of an aluminosilicate based mycotoxin sequestering agent. Broilers consuming the contaminated diet had reduced humoral immune response and lower numbers of mitotic cells in the bursa. Additionally, the birds had higher relative liver weights and microscopically the birds showed alterations of the liver and kidneys. The addition of the sequestering agent did not alleviate any of the negative effects associated with ochratoxin A consumption.

HSCAS (Novasil®)

A hydrated sodium calcium aluminosilicate (HSCAS) from natural occurring montmorillonite clay (Novasil™, Engelhard Corp., Cleveland, OH) is the most widely studied mycotoxin-sequestering agent among the mineral clays. Interest in its potential as a mycotoxin sequestrant was based on ability of the silicate mineral to sequester positively charged or cationic compounds (*47*). Two early studies (*48, 49*) identified this product, which is sold as a feed additive for reducing moisture in feedstuffs and improving flow properties during storage and transport, as also being effective in reducing aflatoxicosis in poultry. Based on a series of *in vitro* screening tests of several silicate minerals Phillips *et al.* (*49, 50*) determined that this HSCAS was the most effective mineral clay for aflatoxin sequestration due to its high affinity for and stable association with aflatoxin B_1. Later on, Phillips *et al.* (*51, 52*) determined that the β-carbonyl portion of the aflatoxin molecule bound to the uncoordinated edge site containing aluminum ions in this HSCAS. Moreover, the authors determined that the maximum quantity of aflatoxin that could be bound was 200-232 nmol

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per milligram of HSCAS and that the reaction reached equilibrium after 30 minutes. What followed were a series of experiments that provided evidence of the potential of this HSCAS as an aflatoxin sequestering agent. Ramos and Hernandez (*47*) reviewed over 20 publications, which demonstrated the *in vivo* capacity of this aflatoxin sequestering agent in poultry (chickens and turkeys), swine, ruminants (dairy cattle, lambs, and goats), and mink.

As this HSCAS was able to sequester aflatoxin in several animal models, its ability to bind other mycotoxins has also been studied. Bursian *et al.* (*53*) showed that although addition of 0.5% NovaSil® to a diet contaminated with ZEA did not alleviate the estrogenic effect of the toxin, it did mitigate some of the adverse effects of ZEA on reproductive performance of female mink. Similarly, Huff *et al.* (*54*) concluded that addition of 0.5% HSCAS to a diet contaminated with ochratoxin A and aflatoxin, alone or combination, had little effect on toxicity caused by either ochratoxin A alone or the combination of aflatoxin and ochratoxin A. Kubena *et al.* (*55*) conducted a series of experiments in which the HSCAS, at 0.5% of the diet, showed no reduction in T-2 toxin and diacetoxyscirpenol toxicosis in broilers.

Based on the identified mechanism for the formation of the aflatoxin-HSCAS complex, it is not unexpected that this product has been shown to be ineffective in adsorbing other non-polar mycotoxins.

Yeast Cell Wall-Derived Sequestering Agents

Initial research with a yeast cell culture preparation based on a *Saccharomyces cerevisiae* strain 1026, in which performance was improved in poultry (*56*), led researchers to hypothesize that the yeast culture had the ability to sequester mycotoxins. Studies with whole yeast cells led to identification of specific cell wall components in yeast that interacted with mycotoxins. Modifications in manufacturing techniques have allowed the production of a specific modified yeast cell wall preparation with the potential to adsorb a range of mycotoxins (*57*). This preparation, commercially available as Mycosorb® (MS) (Alltech Inc., Nicholasville, KY, USA), has been studied in detail with over 20 published reports in the scientific literature on both mode of action and animal response. The adsorptive capacity of the carbohydrate complexes in the yeast cell wall offers an interesting alternative to inorganic adsorbents. Mineral clay-based compounds and activated carbon are generally used at high concentrations (>0.5% of the diet) in animal feeds. In animals such as broilers and young pigs, where feed intake may limit economic performance, it is important that non-nutritive feed additives take up as little space in the diet as possible. Additionally, high levels of inclusion could provide excessive sequestrant capacity that may decrease the bioavailability of important micronutrients. In contrast, the high toxin affinity and adsorptive capacity of the

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yeast cell wall-derived adsorbents allow use at much lower concentrations (<0.1% of the diet). Since these preparations can be used at low concentrations (0.5-2 kg/T), their inclusion would have minimal impact on nutrient density of the diet.

Mode of Action

In vitro studies have helped to characterize the interactions between the yeast cell wall-derived adsorbent and specific mycotoxins. Serial elution of the aflatoxins adsorbed to yeast glucomannan clearly demonstrated that the adsorption of the mycotoxin is a concentration-dependent reversible process and that aflatoxins are not modified during adsorption (*11*). This is important, since it clearly established the basic kinetic mechanisms associated with adsorption. Aflatoxin adsorption is also influenced by the relative acidity (pH) in the aqueous environment. Maximum adsorption occurs at a pH of approximately 4.0. In addition, adsorption is also influenced by the relative phosphate concentration in an aqueous environment. Maximum adsorption was observed in a buffer that contained 0.5 M phosphate. Both pH and phosphate concentration optima are consistent with those found in the gastrointestinal tract and suggest that the conditions in the gastrointestinal tract would enhance adsorption and not decrease the mycotoxin-adsorbent interactions.

Recently a series of experiments have utilized ZEA to help explain the mode of action in the formation of the mycotoxin-MS complex. The kinetics of adsorption can be described using a concentration-dependent model (Figure 1). Yiannikouris *et al.* (*58, 59*) determined that two types of interaction were responsible for the formation of the complex. These consist firstly of stable interactions between glucan and toxin molecules, and secondly of less stable glucan-toxin interactions. Adsorption is associated with β-D-glucans in yeast cell wall material; and weak non-covalent bonds are involved in the formation of the ZEA-cell wall complexes. This indicates that the chemical interaction between β-D-glucans and ZEA is not the result of covalent linkage but is better described as ***toxin adsorption***. Nuclear Magnetic Resonance (NMR) and molecular modeling studies showed that the hydroxyl groups of ZEA are involved in these interactions and that there is geometric complementarity between ZEA and the single helix of β-(1,3)-D-glucans (Figure 2). Modeling studies were further utilized to confirm the NMR data and made it possible to visualize the formation of hydrogen bonds involving not only the hydroxyl groups but also the lactone and ketone groups of ZEA, which interact with the β-D-glucopyranose units of β-(1,3)-D-glucans. The authors also utilized molecular mechanic analyses to help confirm the existence of less stable interactions, called 'stacking' interactions, involving van der Waals forces between the aromatic ring of ZEA and the ring structures of β-D-glucopyranose units.

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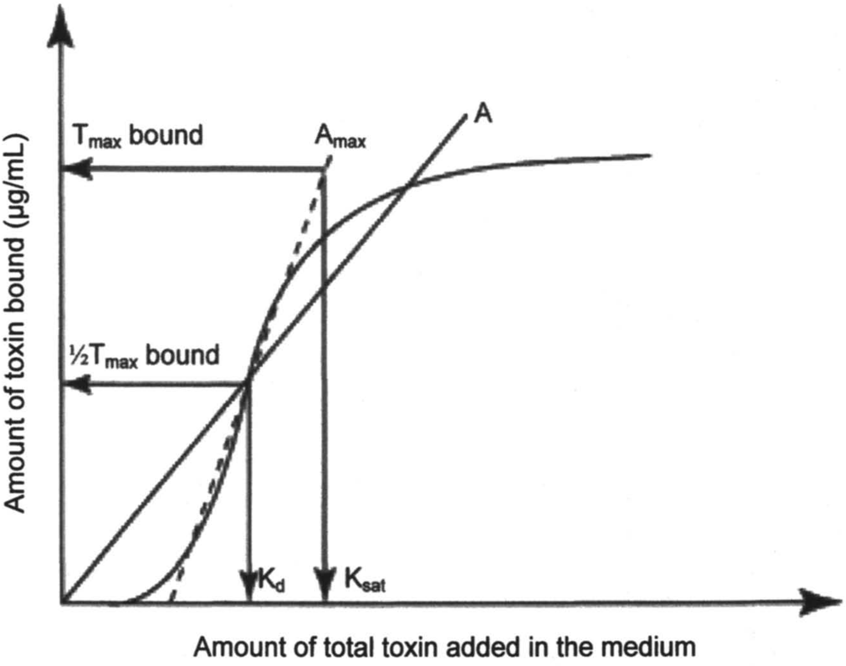

Figure 1. Adsorption curve demonstrating concentration-dependent interaction of the active β-glucan fraction of yeast with a mycotoxin. K_{sat} = saturation point (µg/mL), A = affinity rate (%), and A_{max} = maximal affinity (%).

Analysis of the side groups of some β-(1,6)-D-glucan units allowed the authors to correlate macroscopic observations *in vitro* with adsorptive capacity, which confers on these molecules the ability to stabilize the complex formed. These chains increase the stability of the complex formed between ZEA and β-(1,3)-D-glucan by increasing the contribution of van der Waals forces to the potential energy of the complex. The physical and chemical properties of the yeast cell wall determine its ability to adsorb mycotoxins. Additionally, the highly complex organization of the yeast cell wall increases the adsorptive ability of the glucans.

In respect to aflatoxin and other mycotoxins Yiannikouris et al. (*57*) reported that single helical structure of the β-(1,3)-D-glucans was important in the formation of a mycotoxin (aflatoxin B_1, deoxynivalenol (DON), and patulin) complex. The researchers concluded that the interaction between the β-(1,3)-D-glucans and the mycotoxins is driven by steric complementarities that enable an important participation of van der Waals interactions causing some stacking effects as well as stable intermolecular hydrogen bonding the hydroxyl, lactone,

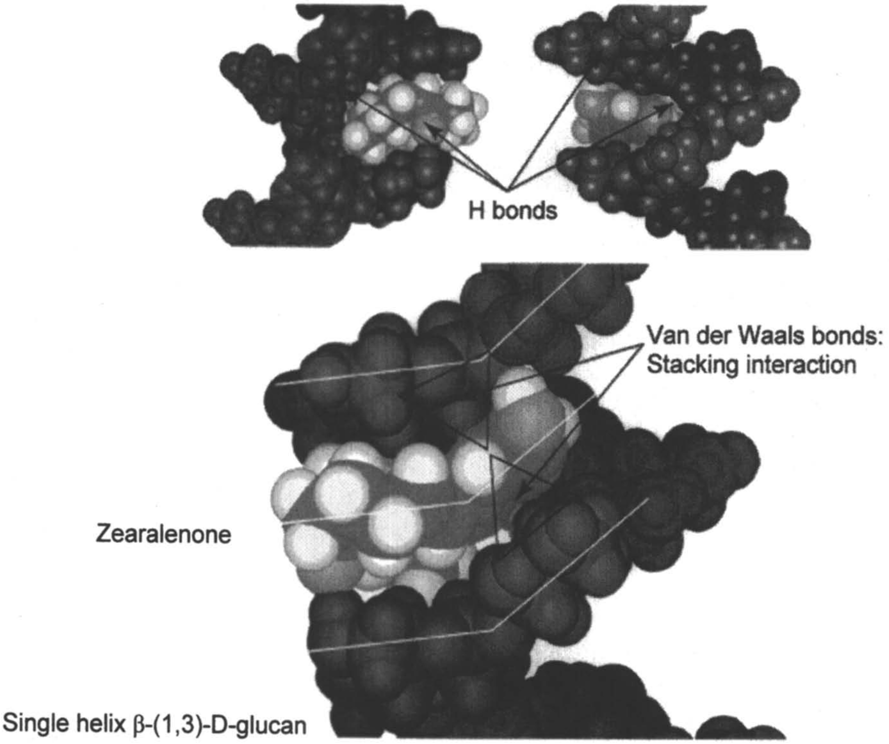
H bonds
Van der Waals bonds:
Stacking interaction
Zearalenone
Single helix β-(1,3)-D-glucan

Figure 2. Computer-generated view of the energy-minimized structure of the docking of the most favorable conformation of zearalenone into the single helix of β-(1,3)-D-glucan chain. Arrows indicate hydrogen bonds. Lines highlight the steric complementarity between zearalenone and β-(1,3)-D-glucan. (Adapted from Yiannikouris et al. (60))

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and ketone groups present in many mycotoxins. They however caution that not all mycotoxins are bound equivalently. Moreover, the researchers commented that environmental conditions like pH and temperature are important in the stability of the toxin-sequestering agent complex. Since van der Waals attraction and hydrogen bonds can occur throughout the gastrointestinal tract with other components of the diet (*i.e.*, minerals and vitamins) further dosimetry data is needed to understand interactions between the sequestering agent and these ingredients. Regardless of these reports *in vitro* findings must be further complemented by *in vivo* data.

Animal Trials

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Supplementation with the yeast glucomannan polymer MS has been found to be beneficial in reducing the individual and combined adverse effects of several mycotoxins in poultry. Raju and Devegowda (*61*) demonstrated that the inclusion of MS improved the body weight gains and antibody titres suppressed by aflatoxin, ochratoxin A, and T-2 toxin. The polymer also improved serum biochemical and hematological parameters. Similarly, broilers consuming a mixture of mycotoxins (168 ppb aflatoxin, 8.4 ppb ochratoxin A, 54 ppb ZEA and 32 ppb T-2 toxin) had significantly lower body weight and feed consumption, which were alleviated by the inclusion of MS at 0.05% of the diet (*62*). Dvorska and Surai (*63*) evaluated the antioxidant status of quail consuming T-2 toxin plus either a zeolite (3% of diet) or MS (0.1% of the diet). The authors concluded that, unlike the tested zeolite, the glucomannan polymer was able to significantly inhibit liver antioxidant and vitamin A depletion caused by the T-2 toxin. They stated that toxin adsorption by the polymer could be inhibiting toxin absorption from the intestine as well as preventing the participation of T-2 toxin in development of oxidative stress in the intestine. Due to antioxidant depletion in the liver, susceptibility to lipid peroxidation increased more than 2-fold in the test animals. Inclusion of MS in the T-2 toxin-contaminated diet significantly decreased tissue susceptibility to lipid peroxidation in comparison to diets containing toxin only, although the inclusion of the MS was unable to completely mitigate the powerful stimulating effect of T-2 toxin on lipid peroxidation.

Aurofusarin, a secondary metabolite of *Fusarium graminearum*, is responsible for significant changes in fatty acid and antioxidant profiles of quail eggs. The inclusion of MS in the diet of quail consuming aurofusarin prevented changes in fatty acid and antioxidant composition in the egg yolk as well as helped to maintain vitamin E concentration in the egg yolk and liver of the newly hatched quail (*64*). Furthermore, retinol, retinyl esters, and carotenoids in the liver of newly hatched chicks from eggs contaminated with aurofusarin were also increased due to MS in the diet. Consequently, lipid peroxidation in tissues of newly hatched quail was significantly reduced (*65*).

Swamy *et al.* (*66*) noted that a blend of *Fusarium* toxins in contaminated corn and wheat (up to 8.3 mg DON/kg) in diets fed to slow-growing broiler chickens resulted in growth depression only in the finisher period. Addition of MS at 2.0 kg/T of feed partially prevented the growth depression. A subsequent study of more rapidly growing broilers indicated that feeding contaminated grains depressed growth and feed intake in the grower period and that this could also be prevented by supplementation with the MS yeast cell wall polymer (*67*). Raju and Devegowda (*68*) fed broilers diets containing aflatoxin (2 mg/kg) and T-2 toxin (1 mg/kg). Broilers consuming the mycotoxin-contaminated diets had significantly reduced feed intake, weight gain and feed efficiency, weights of thymus and bursa of Fabricius, antibody titers against Newcastle disease, and infectious bursal disease. The MS glucomannan (1 kg/T of feed) significantly improved body weight, feed intake, antibody titers, weights of the thymus and the bursa of Fabricius.

Chowdhury and Smith (*69*) evaluated the effects of feeding laying hens diets naturally contaminated with several *Fusarium* toxins with or without the inclusion of MS. The authors found an initial reduction in feed intake (month 1), which was followed by increased feed intake (months 2 and 3) and large reductions in the utilization of feed for egg production. Supplementation with 2.0 kg MS/T prevented these effects as well as adverse effects on egg production, egg shell weight, blood uric acid concentrations and biliary immunoglobulin A concentrations.

Starter pigs (initial body weight 10 kg) fed graded levels of *Fusarium* toxin-contaminated grains for three weeks had depressed weight gain and feed intake in the first week of the experimental period when approximately 4.6 mg DON/kg was fed (*70*). Supplementation with 2.0 kg MS/T could not prevent the severe depression in growth rate and feed consumption. However, MS did prevent some of the mycotoxin-induced neurochemical changes, including depression of norepinephrine concentration in the pons. The depression in norepinephrine concentration was thought to be caused by the mycotoxin fusaric acid. Hence it is possible that the glucomannan polymer is effective in reducing its absorption from the intestine. MS addition also prevented elevation in blood concentrations of immunoglobulins A and M in pigs fed contaminated grains. In a similar study with starter pigs, the authors concluded that the greatest factor contributing to reduced growth rate when *Fusarium* toxin contaminated grains were fed was reduced feed intake (*71*). Although supplementation with yeast cell wall polymer prevented some of the toxin-induced changes in metabolism, the feeding of 2.0 kg MS/T could not completely prevent appetite suppression.

More recently a series of experiments have further evaluated the utilization of this yeast cell wall polymer as an effective *in vivo* mycotoxin sequestering agent. Broiler breeder hens fed diets containing aflatoxin (3 mg/kg) had significant negative effects for egg production, percentage hatchability, and embryonic mortality (*56*). These effects were significantly improved by the inclusion of the yeast cell wall polymer. Similarly, Karaman *et al.* (*72*) saw both

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slight and moderate improvements in pathological lesions when either 0.5 g/kg or 1 g/kg of the yeast cell wall polymer were fed to broiler chicks consuming 2 mg/kg of aflatoxin.

Poultry consuming diets contaminated with several *Fusarium* toxins and who had significant changes in neurotransmitter ratios saw significant increases in 5-hydroxytryptamine and 5-hydroxyindoleacetic acid concentrations when the yeast cell wall polymer was included in the diet (*73*). Production losses associated with the *Fusarium* mycotoxins however were significantly alleviated by the polymer. Chowdhury and Smith (*69*) fed layers diet contaminated with *Fusarium* mycotoxins with or without the inclusion of the polymer. Egg production and egg mass were affected in the first half of the 12 wk experiment independent of the inclusion of the adsorbent. Effects on plasma uric acid concentrations and relative kidney weights were however alleviated by the inclusion of the polymer. The same researchers found that the inclusion of the polymer in turkey diets was effective in preventing the minor effects on blood cell counts (basophil and monocyte) caused by the consumption of *Fusarium* mycotoxins (*74*). The authors cautioned that because of the minor effects observed, the changes observed with the inclusion of the polymer where not sufficient to make conclusive remarks on the effectiveness of the sequestering agent. Recently, the same authors (*75*) saw a significant improvement by the polymer in the B-lymphocytes in peripheral blood and in bilary IgA concentration of layers consuming diets contaminated with DON (12 mg/kg), 15 acetyl-DON (0.5 mg/kg), and ZEA (0.6 mg/kg).

Although considerable information is available supporting the effectiveness of this polymer in alleviating the effects of feeding diets contaminated with different and multiple mycotoxins and in many different experimental species. However, evaluation of mycotoxin specific bio-markers is still lacking.

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Multi-component Sequestering Agents

Several products that claim to degrade mycotoxins have recently been studied; they belong to different classes of sequestering agents combined with enzymatic components. Dänicke *et al.* (*76*) measured ZEA and its metabolites in blood and bile fluids of broilers administered a bolus containing approximately 6 mg ZEA/kg body weight with or without the addition of a multi-component sequestering agent (Mycofix-Plus®). The authors concluded that the sequestering agent had minor or no effect on ZEA and ZEA metabolite concentrations in the samples analyzed. Similarly, Dänicke *et al.* (*77*) found no benefit in the utilization of the same multi-component sequestering agent in diets of broilers containing varying levels of *Fusarium*-produced mycotoxins (primarily DON and ZEA). The same product was found to be ineffective in alleviating the effects caused by diets containing multiple *Fusarium*-produced mycotoxins on growing bulls (244 kg) (*78*). More recently, Dänicke *et al.* (*79*) saw no positive reduction in DON concentrations in serum or no positive effect in production in growing pigs consuming approximately 2.7 mg/kg of DON during the starter period and 2.8 mg/kg of DON during the grower period. The authors concluded that the sequestering agent was ineffective in adsorbing the *Fusarium* produced mycotoxins and thus reducing their intestinal absorption.

Future Research Needs

Further research on the metabolism and mechanisms of mycotoxins will allow for further *in vivo* studies to examine more closely the effectiveness of sequestering agents by measuring specific biochemical markers (biomarkers). Currently biomarkers for fumonisin (sphingoid bases), aflatoxin (aflatoxin M_1 residues in milk and aflatoxin/ DNA or protein adducts), and ZEA (metabolites and glucuronide conjugates) are known, although methods for their analyses are not always readily available and tend to require expensive and complex methods. Other non-specific biomarkers like neurotransmitters (trichothecene mycotoxins) have shown effectiveness for evaluation of a sequestering agent.

Many commercially available sequestering agents have only basic *in vitro* experiments to support their claims as mycotoxin treatments. Additionally, since many of these products are often registered as flow and anti-caking agents they are not regulated by governmental agencies for the purpose for which they are used in the field. Independent research institutes and universities have however, set out to investigate these products and to make information available to the general public.

Although most of the sequestering agents are considered GRAS, long term safety and other toxicological studies are still lacking and should be conducted to determine that no toxicity or nutrient-interaction occurs at the animal level, especially when these products are recommended for daily use.

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Conclusions

Mycotoxin contamination of crops and the ensuing consumption of these contaminated ingredients by animals are inevitable parts of animal production systems. The most widely utilized strategy available to reduce or impede this exposure is the addition of materials that sequester the toxin and inhibit intestinal toxin absorption. The available data suggest that care must be taken when examining potential sequestering agents. *In vitro* tests, although informative, should not be used as the sole indicator of sequestrant efficacy. Additionally, generalization should be avoided, as sequestrant compounds differ in efficacy even within the same category. Moreover, the chemical complexity of mycotoxins means that the effectiveness of a compound in sequestering one mycotoxin does not mean equal ability to sequester other mycotoxins. Therefore, selection of a mycotoxin sequestering agent must be based on published scientific data, gathered under controlled scientific conditions, with the target animal species exposed to the levels and varieties of mycotoxin contamination seen under practical conditions.

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Chapter 8

Molecularly Imprinted Polymers for Mycotoxins

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Molecularly imprinted polymers (MIPs) are a class of synthetic receptors capable of selective recognition of analytes. Recent developments in imprinting technology have made possible several practical applications for these materials, including the use of MIPs in mycotoxin detection. Structure-activity relationships of reported MIPs for ochra-toxin A, deoxynivalenol, and zearalenone are reviewed. In addition, results from a molecularly imprinted solid phase extraction (MISPE) analysis of a MIP designed to recognize moniliformin show the binding properties of the moniliformin MIP are influenced by solvent and matrix effects.

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Mycotoxins are a structurally diverse class of natural products with potential to contaminate agricultural commodities. These secondary metabolites of fungal origin are associated with a variety of adverse effects in animals and humans, including, effects on reproduction, as well as carcinogenic, immunotoxic, hepatoxic, genotoxic, and nephrotoxic effects (*1*). Although significant efforts are placed on control and prevention, the potential risks of mycotoxin contamination require trace level monitoring. Furthermore, assurances of mycotoxin science and mycotoxin regulation are limited by the reliability and accuracy of detection levels. Sampling procedures address reliability and additional improvements in mycotoxin detection have been achieved through the application of selective binding materials for mycotoxins, such as the use of antibodies. Successful examples of the use of antibodies for mycotoxin detection include enzyme linked immunosorbent assays (ELISAs) and immunoaffinity columns. Applications of antibodies and rapid methods for mycotoxin analysis have been recently reviewed (*2-4*).

More adaptable selective binding materials have potential to improve mycotoxin detection. Molecularly imprinted polymers (MIPs) are selective binding materials capable of high capacity, favorable activities in organic solvents, relative low cost of synthesis, and improved chemical and physical stability (*5-7*). Recent advances in molecular imprinting technology have brought about practical applications of MIPs as recognition components in formats such as molecularly imprinted solid phase extraction (MISPE) clean-up methods, ELISA-type assays, and sensors (*8-13*).

MIPs: Overview

The molecular recognition properties of MIPs are attributed to binding sites formed about template molecules during polymer synthesis. Successful imprinting produces polymers with selective recognition of analytes. Types of interactions associated with MIPs are similar to those of natural receptors, such as solvent effects (hydrophobicity), favorable electrostatic interactions, hydrogen bonding, and shape selectivity. The basic concepts behind molecular imprinting are not new to chemistry, and a review of the history of the field has been published (*14*). In addition, there are several recent, in-depth reviews of imprinting technology (*5-13, 15*).

Molecular imprinting can be carried out by a variety of methods, such as, covalent imprinting, semicovalent approaches, or the use of silicas. Most modern imprinting is performed through a process called non-covalent imprinting (*5-7*). An emphasis of non-covalent imprinting is the formation of a pre-polymerization complex from which binding sites are formed. A schematic diagram of noncovalent MIP synthesis is outlined in Figure 1. In this process, a template

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molecule interacts with a functional monomer to form a pre-polymerization complex in the presence of a solvent. The solvent is known as the porogen because of its role in pore forming during polymerization. A crosslinking monomer is added to the pre-polymerization complex to form a scaffold around the binding site during polymer synthesis. Often an initiator is added to promote polymerization. The most common chemistry for imprinting is free radical polymerization, either through photolytic or thermolytic conditions. Following polymer synthesis, template is removed from the polymer through a variety of methods, including Soxhlet extraction, sonication, liquid extraction, microwave assisted extraction, or pressurized liquid extraction (*16-18*). The result is a highly crosslinked polymer with exposed imprinted cavities capable of analyte binding. A non-imprinted polymer (NIP) is typically synthesized in parallel following an identical procedure without template.

The challenge of successful imprinting is the selection of parameters for sufficient activity of the imprinted material in the desired application. Parameters include the reagents, stoichiometry, and synthetic conditions. Solvent properties, such as dielectric constant and solvent capacity, are significant for selection of the porogen and performance of the crosslinking monomer. Selection of components of the pre-polymerization complex (template and functional monomer) is vital for successful imprinting. Further advancements in molecular imprinting technology may be related to improved understanding of these complexes (*19*). It should be noted that binding sites resulting from non-covalent imprinting are complicated by the formation of heterogeneous populations of binding sites with varying affinities, as well as, the role of molecular shape and potential competing template-template interactions on binding site formation and the recognition properties of the resulting polymers (*20-23*).

An obvious solution for template selection is the use of the analyte. However, concerns with the analyte, such as toxicity, cost, stability, and the potential for interferences in analysis by bleeding of residual template (analyte), can complicate the use of a mycotoxin imprinted MIP. The use of toxin analogs as templates addresses many of these issues. Analogs as templates have been successfully applied in molecular imprinting (*16, 24*).

Whether the imprint molecule is an analog or analyte, the functional groups of the functional monomers and analyte are the focus of binding interactions of the MIP; therefore the selection of functional monomer is key to obtaining required activity from a polymer. Ideally, the functional monomer should be selected from the best activity in the intended application of the MIP. However, with a large number of available functional monomers and difficulties of MIP preparation and evaluation, more economical and less labor intensive methods for functional monomer selection have been developed. These predictive methods include the use of computational modeling of pre-polymerization complexes (*19, 25-28*). In addition, spectroscopic techniques have been

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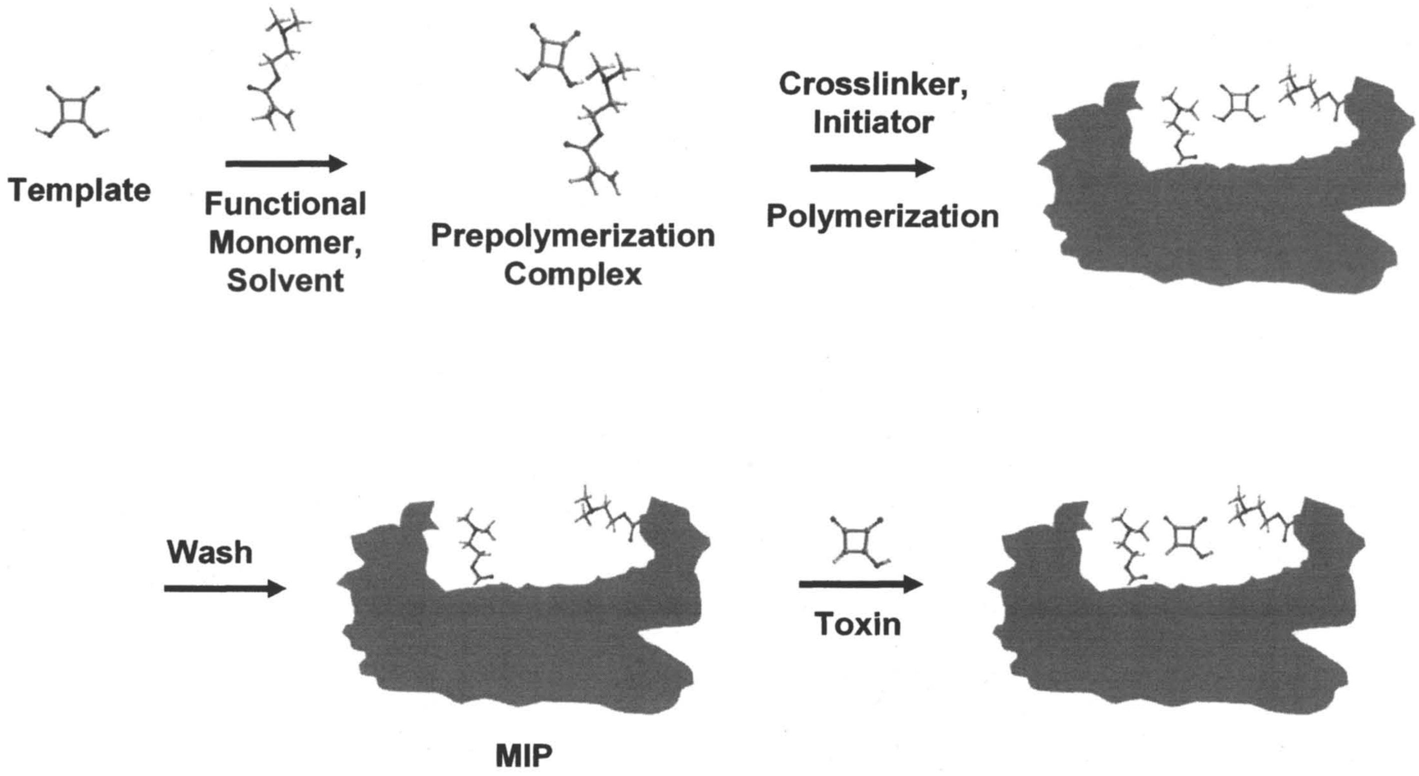
Template
Functional Monomer, Solvent
Prepolymerization Complex
Crosslinker, Initiator
Polymerization
Wash
MIP
Toxin

Figure 1. Schematic diagram of molecularly imprinted polymer synthesis.

explored, such as the use of nuclear magnetic resonance (NMR) spectroscopy and ultraviolet (UV) spectroscopy to experimentally probe pre-polymerization complexes (*6*, *29-31*).

Applications of successfully imprinted materials include solid sorbents for purification and isolation, ligand screening, synthetic catalysts, and as sensors (*8-13*, *32-34*). MIPs for mycotoxins have been applied in MISPE methods. MISPE columns apply MIPs as solid sorbents for the preconcentration and clean-up of extracts.

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Ochratoxin A MIPs

Ochratoxin A (**1**) is produced by *Aspergillus* and *Penicillium* species and is a natural contaminant of cereals (see Figure 2). Ochratoxin A is the most studied and first mycotoxin that imprinted polymers have been synthesized to recognize. Its structure consists of phenylalanine and dihydroisocoumarin moieties, with the carboxylic acid and phenolic hydroxyl functional groups capable of forming favorable binding interactions with functional monomers. A recent structural analysis of ochratoxin A with empirical modeling identified favorable intramolecular interactions involving the phenolic hydroxyl (*35*). These types of competing intramolecular interactions are important considerations and have the potential to influence recognition.

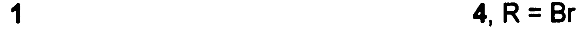

Figure 2. Ochratoxin A, 1, and templates, 2-4, for imprinting MIPs.

The first MIPs for ochratoxin A were imprinted with a toxin analog, *N*-(4-chloro-1-hydroxy-2-naphthoylamido)-L-phenylalanine (*36*). The functional monomer was methacrylic acid and polymers were evaluated by LC analysis using the polymers as solid phase packing in LC columns. Greater retention and an imprinting effect were observed for the template and ochratoxin A in the MIP packed column. By investigating the retention of several structural analogs, the authors explain that favorable hydrogen bonding interactions and steric factors contribute to recognition of ochratoxin A by the MIP compared to the NIP packed column.

Polymers imprinted with ochratoxin A have been designed using a computational approach to select the best functional monomer (*37*). Polymers synthesized with basic functional monomers 1-vinylimidazole and 2-(diethylamino)ethyl methacrylate produced imprinted polymers with continuous bleeding of the ochratoxin A template. The continuous bleeding of ochratoxin A prevented the use of the polymer for ochratoxin A analysis. However, a MIP imprinted with ochratoxin A using methacrylic acid and acrylamide as functional monomers exhibited less template bleeding. The binding properties of the methacrylic acid and acrylamide polymer were characterized by MISPE analysis. Optimal binding conditions of the MIP were found to be under aqueous conditions at high buffer concentration and a pH lower than 7. Addition of acetonitrile (10%) reduced the ochratoxin A binding by 70%. Binding of ochratoxin A by the polymer was influenced by loading solvent and differences in ochratoxin A binding were possibly due to swelling and shrinking of the polymer by solvent.

Ochratoxin A analogs and novel functional monomers were used successfully to obtain selective ochratoxin A binding MIPs (*38*). Novel templates displayed in Figure 2 were applied for imprinting, including the unsubstituted (**2**), and 4-chloro (**3**) and 4-bromo (**4**) derivatives. Novel functional monomers included the basic quinuclidin-3-yl methacrylamide and quinuclidin-3-yl methacrylate. Additional monomers included *tert*-butyl methacrylamide and *tert*-butyl methacrylate. Initial evaluations consisted of chromatographic evaluation of MIP and NIP packed LC columns and frontal analysis. Promising polymers were investigated as sorbents for the clean-up of ochratoxin A from red wine (*39*).

The effects of functional monomer selection on the binding properties of MIPs imprinted with ochratoxin A have been investigated by binding assays (*40*). Ochratoxin A binding was related to pK_a of the functional monomer and the MIP synthesized with *N*-phenylacrylamide possessed the greatest affinity for ochratoxin A in the investigation. In addition, the MIP material was applied as a sorbent for MISPE analysis of ochratoxin A from wheat extracts (*41*). The developed method had a detection limit of 5.0 ng mL^{-1} for an analysis that could be completed in five min.

Polypyrrole films imprinted with ochratoxin A synthesized through electropolymerization have been employed as recognition components in surface plasmon resonance devices (*42*). In addition, polypyrrole stainless steel frits have been imprinted with ochratoxin A and applied for the online preconcentration of this mycotoxin (*43*). Incorporation of carbon nanotubes on frits during imprinted electropolymerization resulted in an imprinted polypyrrole frit selective for ochratoxin A with a limit of detection of 12 ppt and a limit of quantitation of 41 ppt (*44*).

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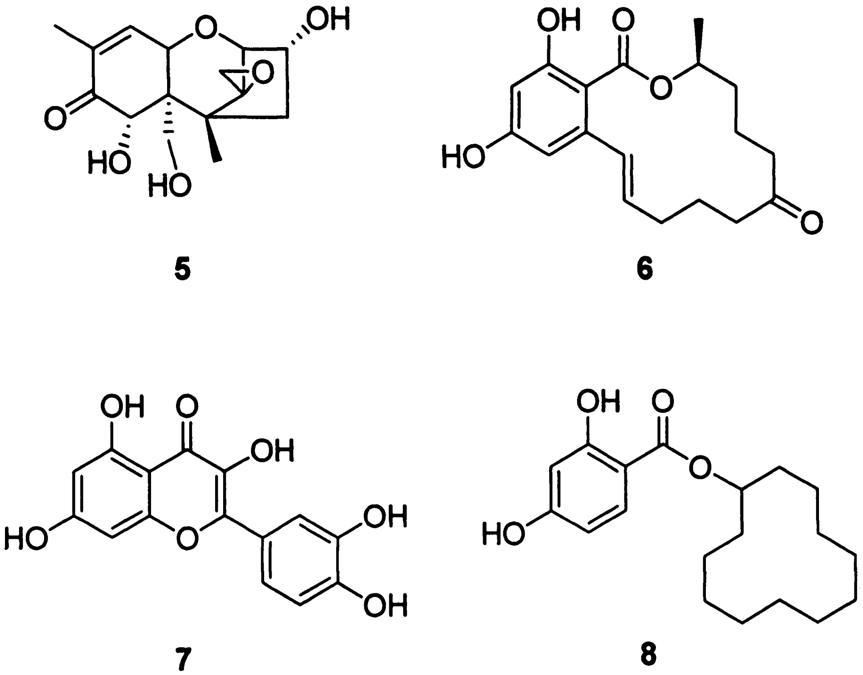
Deoxynivalenol MIPs

Deoxynivalenol (5), also known as vomitoxin, is produced by ***Fusarium*** species, including ***F. graminearum*** and ***F. culmorum***, as well as other fungal species (see Figure 3). MIPs imprinted with deoxynivalenol have been prepared and characterized as stationary phases in MIP and NIP packed LC columns (*45*). To address the issue of expense of commercially available deoxynivalenol, polymers were imprinted with deoxynivalenol obtained from culture. Polymers were evaluated not only for their activity with the template, deoxynivalenol, but also congeners nivalenol, fusarenon-X, 3-acetyl deoxynivalenol, and 15-acetyl deoxynivalenol.

MIPs using both acidic (methacrylic acid) and basic (4-vinylpyridine) functional monomers exhibited imprinting effects. The retention indexes for deoxynivalenol and nivalenol were higher than that of fusarenon-X, 15-acetyl deoxynivalenol, and 3-acetyl deoxynivalenol. The lower activity of the acetylated derivatives of deoxynivalenol was attributed to the prevention of favorable hydrogen bonding of C-15 and C-3 hydroxyls.



5 6 7 8

Figure 3. Deoxynivalenol (5), zearalenone (6), and zearalenone analogs (7-8) for imprinting MIPs.

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Zearalenone MIPs

Zearalenone (**6**), is a *Fusarium* mycotoxin with potential to contaminate cereal grains and is associated with estrogenic activity. This phenolic resorcyclic acid lactone contains a flexible 14 atom cyclic ring system featuring a double bond (see Figure 3). Attempts to imprint with zearalenone by free radical polymerization produced polymers with limited recognition (*45*). Polymer synthesis may be complicated by the flexibility of zearalenone and the potential for the unsaturated ring of zearalenone to react with other reagents resulting in a polymer with binding sites occupied by covalently linked zearalenone. These issues were addressed by using a toxin analog, quercetin (**7**), to imprint polymers and evaluating the zearalenone binding properties of the resulting polymers by LC analysis. Retention time increased by 50% for zearalenone evaluated with the quercetin imprinted MIP with 4-vinylpyridine as the functional monomer compared to the NIP. This retention was greater than that of the zearalenone imprinted MIP. The quercetin imprinted MIP has been applied for the clean-up and detection of zearalenone contamination in beer (*4*).

MIPs for zearalenone have been prepared using cyclododecyl-2,4-dihydroxybenzoate (**8**), as a rationally designed template based on octanol/water partition coefficients (*18*). Template was removed by a pressurized liquid extractor. Initial evaluation by frontal analysis identified 1-allylpiperazine as the best functional monomer over 2-(diethylamino)ethyl methacrylate, 4-vinylpyridine, and 2-hydroxyethyl methacrylate. Trimethylolpropane trimethacrylate (TRIM) was used as the crosslinker and acetonitrile was the porogen. Evaluation was carried out in acetonitrile. Cross selectivity was evaluated through the retention of eight structural analogs of zearalenone by the MIP and NIP of the best imprinted material determined by frontal analysis (1-allylpiperazine functional monomer). Compounds that were related to the template by chemical structure and size were better retained compared to smaller compounds with similar functional groups (resorcinol and resorcylic acid). Addition of methanol to the mobile phase further decreased retention, which the authors suggest is due to competing hydrogen bonding of the polar protic methanol.

The cyclododecyl-2,4-dihydroxybenzoate imprinted MIPs have been applied for the MISPE clean-up of zearalenone and α-zearalenol from cereal grain extracts (*46*). Recoveries ranged between 85-97% and the method has been validated on a corn reference.

Future Developments in MIPs for Mycotoxins

With hundreds of known mycotoxins and recent advances in MIP technology, prospects for future applications of MIPs to improve mycotoxin

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detection are promising. Recent efforts report selective recognition by MIPs for other important mycotoxins. A MIP imprinted with aflatoxin B_1 using allylamine and hydroxyethyl methacrylate as the fuctional monomers was able to recognize aflatoxin B_1 in corn matrix using a MISPE column (*47-48*).

Utilization of imprinted synthetic receptors in pre-existing analytical methods has been demonstrated for a structurally diverse set of analytes. Further application of MIPs for mycotoxin detection should address shortcomings of existing technologies, such as the low binding capacity or other limitations of antibodies. An example is the recent work with moniliformin, a small molecular weight mycotoxin for which selective antibody development has been difficult. MIPs for moniliformin were synthesized as an alternative to antibody development (*49*). Two analogs were investigated for imprinting the moniliformin MIPs (see Figure 4). The optimal ratio of reagents for moniliformin binding depended on the imprinting template. Although binding assays were the method for selecting the optimal polymer composition of the moniliformin MIPs, the materials had an imprinting effect in a MISPE analysis and show potential for further development. A more thorough study of the moniliformin MIP as a sorbent in MISPE columns is presented.

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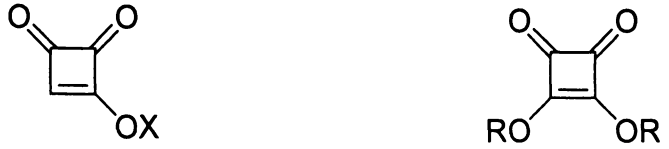
Materials and Methods

All chemicals and reagents were purchased from Sigma-Aldrich (St. Louis, MO). Inhibitors were removed from reagents by column chromatography with the appropriate inhibitor remover packed columns. Extraction reservoirs (1.5 mL) and corresponding polyethylene frits were obtained from Alltech (Deerfield, IL).

MIP Preparation

MIPs were synthesized as previously described (*49*), with the following modifications for scale up. Template, 3,4-diethoxy-3-cyclobutene-1,2-dione (0.5 mmol), anhydrous dimethylformamide (4.5 mL), 2-(dimethylamino)ethyl methacrylate (4 mmol), trimethylolpropane trimethacrylate (20 mmol), and 2,2'-azobisisobutyronitrile (1% reactive bonds) were combined in a 20 mL glass vial fitted with a rubber septum. The vessel was flushed with nitrogen for five min and placed in a 65 °C water bath for 24 h. Non-imprinted polymer synthesis was carried out in parallel without template. Resulting polymers were crushed, then extracted with methanol, water, ethanol, acetonitrile, and acetone via sonication. Polymer fragments were ground in a coffee grinder and wet sieved (acetone). Fine particles were removed from collected fractions (38-75 μm) by repeated sedimentation in acetone (50 mL x 3). Collected fractions (38-75 μm) were dried



9

X = H, Na^+, K^+

10 R = H

11 R = CH_2CH_3

Figure 4. Moniliformin (9) and template (10-11), for moniliformin MIPs.

under vacuum. Further purification of the polymers occurred in the MISPE columns.

LC Analysis

LC analysis was necessary to determine moniliformin levels. The LC system consisted of a Shimadzu LC-20AT pump, SIL-20A autosampler, SPD-M20A diode array detector, a CBM-20A communications bus module, and a Phenomenex Luna 5 μ C18 100 Å column. Instrumentation was controlled by Shimadzu EZstart software. The LC mobile phase consisted of 20% acetonitrile in tetrabutyl ammonium hydrogen sulfate buffer (1.14 mg mL^{-1} of tetrabutyl ammonium hydrogen sulfate and 1.07 mg mL^{-1} monobasic potassium phosphate, pH adjusted to 4.0). Moniliformin concentrations were calculated based on standard solutions using peak areas recorded at 229 nm.

MISPE Experiments

MIP and NIP columns were prepared by packing 25 mg of polymer between two frits in 1.5 mL extract-clean reservoirs. All packed columns were washed with 5 mL of the LC-mobile phase, 30 mL of water, and 5 mL of acetonitrile prior to use. Generally, this was sufficient to remove residual template and other components from the polymer below detection levels. Flow rates were ~ 1 mL min^{-1} using a vacuum manifold. Binding studies were carried out by loading 0.5 mL of standard solutions onto the column. Recoveries were obtained by collecting fractions of a 0.5 mL load of spiked corn extract (10 μg mL^{-1} moniliformin), 1 mL wash with acetonitrile, and two 0.5 mL eluting fractions using the LC-mobile phase. Prior to analysis, loading and washing fractions were diluted 1:1 with LC-mobile phase. Elution fractions for the response curve were measured in the eluting solvent (1 mL), the LC-mobile phase. Corn extract was

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obtained based using a published procedure (*50*). Finely ground corn (5 g) was stirred in 9:1 acetonitrile/water (50 mL) for 30 minutes. After filtration, the filtrate was spiked at the appropriate level of moniliformin from a moniliformin standard (1 mg mL^{-1} in acetonitrile/water). Collected fractions were filtered through PTFE syringe driven filters (0.20 μm) prior to analysis. All experiments were performed in triplicate.

Results and Discussion

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Moniliformin is a cereal grain contaminant produced by several *Fusarium* species. It is a low molecular weight mycotoxin whose mode of action is through the suicide inhibition of the pyruvate dehydrogenase enzymatic system (*51-52*). A variety of methods exists for determination of moniliformin levels, including the use of thin layer chromatography (TLC) and LC-UV (*50, 53-54*). An important consideration in this study was to use existing extraction protocols to load the moniliformin onto the MISPE column and elute with the LC mobile phase for a more rapid determination of moniliformin levels. Moniliformin extraction methods have recently been compared, including the use of aqueous solutions of tetrabutyl ammonium hydrogen sulfate, acetonitrile-water (9:1, v/v), and α-amylase (*55*).

Template selection for this study was based on the small structure of moniliformin and results of previous studies of moniliformin MIPs (*49*). Although 3,4-hydroxy-3-cyclobutene-1,2-dione (**10**), is intuitively a reasonable template to form a pre-polymerization complex, evaluation of the resulting MIPs favored the use of the diethyl derivative (**11**), for the concentration range of interest (~10 μg mL^{-1}). Imprinting effects and sufficient activities were obtained with the template used in this study. The template is larger than moniliformin and does contain the cyclobutenedione moiety to "imprint" a larger moniliformin binding site. This cyclobutenedione moiety may deter the formation of binding sites with unfavorable repulsive interactions from components of the polymer. Eight equivalents of the 2-(dimethylamino)ethyl methacrylate functional monomer were sufficient to obtain desired moniliformin binding activity and maintain an imprinting effect compared to the NIP polymer.

Conditions for the MISPE analysis were selected to emphasize the solvent and matrix effects, rather than retention of moniliformin. Extraction reservoirs (1.5 mL) with 25 mg of polymer were suitable for this purpose. Under these conditions, breakthrough of moniliformin is expected and concerns with breakthrough of the MIP can be addressed by increasing the amount of polymer in the columns.

Initial studies investigated the effects of loading solvent on moniliformin binding to the polymers. In these initial experiments, moniliformin (10 μg mL^{-1})

was loaded (0.5 mL) onto a 25 mg SPE column packed with MIP or NIP in the appropriate extraction solvent. The MIP used in this study was optimized by binding assays to bind moniliformin in acetonitrile, a reported component for moniliformin extraction solvents. Loading moniliformin in a solution of acetonitrile-water (9:1, v/v) bound 96.8% of moniliformin compared to 65.2% of moniliformin bound for the corresponding NIP. However this imprinting effect was lost when loading in another reported extraction solvent, 1% tetrabutyl ammonium hydrogen sulfate, with the MIP and NIP binding nearly all moniliformin. This could be explained by the high concentration of ion pair reagent leaving solution and coating the MIP and NIP irrespective of binding sites. Moniliformin binding was maintained for the MIP when loading in ethanol (95.0% bound); however, a significant decrease in binding was observed in the NIP (47.4% bound moniliformin).

Results for the recoveries of moniliformin from spiked corn extracts are given in Table I. Spiked corn extract (0.5 mL of 10 μg mL^{-1}) was loaded onto a 25 mg SPE column containing MIP or NIP. Binding decreased for both the MIP and NIP comparing the acetonitrile-water (9:1, v/v) binding study to the corn extract study (acetonitrile-water (9:1, v/v)). However, a significant imprinting effect was observed for the MIP in matrix. The NIP bound less than 40% of the moniliformin loaded in matrix, with the MIP binding almost 75%. This imprinting effect was observed in the release of moniliformin using the LC mobile phase as well. The NIP released a greater percentage of bound moniliformin in the first 0.5 mL eluting fraction, while the MIP released significant amounts of moniliformin in both eluting fractions. Labeled standards may be necessary to address issues of recoveries. From the corn extract MISPE experiments, it became apparent that the imprinting effect was present in matrix, and that capacity of the MIP was reduced in matrix.

A concentration-peak area plot was developed for concentrations of moniliformin in spiked corn extract (0.1, 0.5, 1.0, 2.5, 5.0, and 10 μg mL^{-1}). As shown in Figure 5, there is a direct relationship between LC peak area of moniliformin recovered in the eluting fraction (1 mL) from the MISPE clean-up of spiked corn extracts and the moniliformin concentrations of the spiked corn extracts, regardless of breakthrough at 10 μg mL^{-1}.

Shown in Figure 6 are the chromatograms of the eluting fractions (1 mL LC-mobile phase) of a spiked corn extract (0.5 mL of 10 μg mL^{-1} moniliformin) after clean-up on columns packed with MIP and NIP. The columns were washed with acetonitrile (1 mL) following the loading of the spiked corn extract and prior to collection of the eluting fraction. Although both the MIP and NIP are capable of binding moniliformin in the loading and washing steps, and releasing moniliformin in the elution step, the imprinted MIP has a greater capacity for moniliformin. Clean-up of the corn extracts was assisted by the careful selection of loading, washing, and eluting solvents.

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Table I. Recoveries of Moniliformin Loaded in Corn Extract on MISPE and Non-imprinted SPE Columns.

	% Recoveries (mean ± S. D.)	
	NIP	MIP
Load (0.5 mL spiked extract)	62.4 ± 5.4	25.1 ± 0.5
Wash (1 mL acetonitrile)	5.8 ± 0.7	6.3 ± 2.4
Elution (0.5 mL LC mobile phase)	30.4 ± 1.3	45.2 ± 3.1
Elution 2 (0.5 mL LC mobile phase)	4.3 ± 0.1	16.7 ± 1.3
Total	103 ± 5.0	93.3 ± 6.1

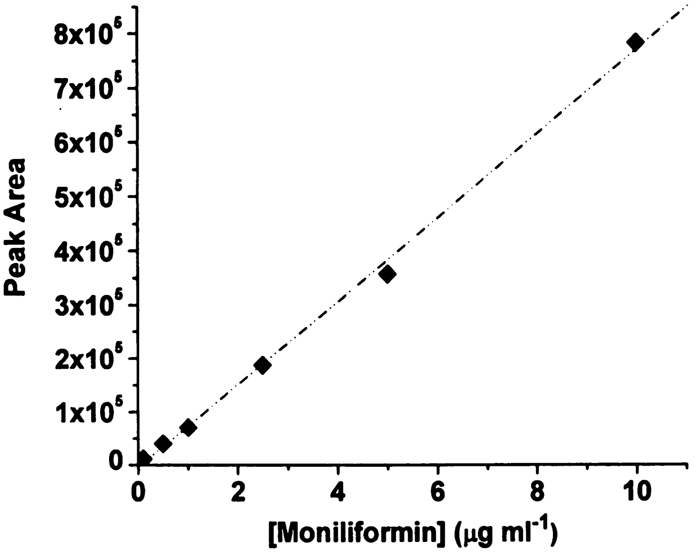

Figure 5. Relationship between peak area of moniliformin isolated by MISPE clean-up of spiked corn extracts and moniliformin concentrations of spiked corn extracts (at 229 nm).

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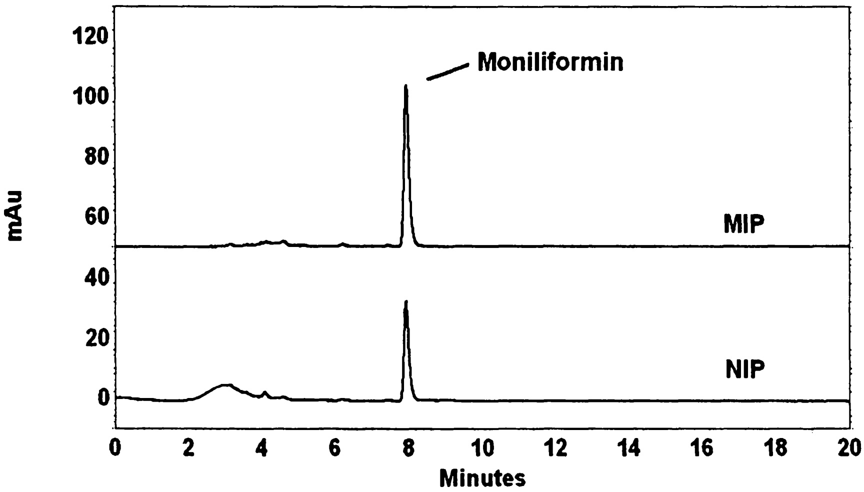

Figure 6. Chromatograms of tmoniliformin recovered from a spiked corn extract following MISPE and non-imprinted SPE clean-up (at 229 nm).

Although the polymers presented here were optimized using binding assays, the MIP had an imprinting effect and was able to bind moniliformin in the MISPE analysis in the presence of matrix. MIP characteristics, such as adaptability and favorable activities in organic solvents, were essential for obtaining a material which could load moniliformin in an extraction solvent, and elute with the LC mobile phase. Providing a more rapid clean-up for analysis is one example of how MIPs can improve mycotoxin detection.

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Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the U.S. Department of Agriculture.

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Chapter 9

Mycotoxins in Alcoholic Beverages and Fruit Juices: Occurrence and Analysis

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A metabolite of black aspergilli growing on grapes, ochratoxin A has often been found in wine on six continents. The European Union (EU) has a regulatory limit of 2.0 ppb in wine and grape juice. Up to 15.6 ppb of ochratoxin A has been found in red wine and 15.3 ppb in a specialty wine (mistelle). Ochratoxin A also occurs in grape juice (up to 6.7 ppb) and frequently in beer in many countries (up to 8.1 ppb). It can be removed experimentally from wine with active dry yeast, yeast lees and fining agents. Aflatoxins have rarely been found in wine but occasionally in beer (ppt levels). Other mycotoxins determined in wine include mycophenolic acid, trichothecin and the *Alternaria* toxins alternariol and alternariol monomethyl ether. Apple juice frequently contains patulin (originating from *Penicillium expansum*), which has also been found in grape and other fruit juices. The *Fusarium* toxins fumonisins and deoxynivalenol (a trichothecene) are additional important contaminants of beer.

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The occurrence of mycotoxins in fruits can lead to contamination of processed food products such as fruit juices and wine, while their occurrence in grains such as barley and corn adjunct can give rise to mycotoxins in beer. The subject of occurrence of mycotoxins in these beverages is very broad. This brief review focuses in more detail on the occurrence and analysis of ochratoxin A (OTA) in wine and fruit juices, a topic of great current interest in those countries where wine is produced, including the EU where there is a maximum allowable limit of OTA in grape juice and wine. Also covered in some detail are occurrence and detection of patulin in apple and in other fruit juices as well as OTA in beer. Background information on the fungal origin and toxicology of those mycotoxins relevant to human health - including carcinogenicity, immunotoxicity, hepatotoxicity, nephrotoxicity, teratogenicity and neurotoxicity - can be found in the report on mycotoxins of the Council for Agricultural Science and Technology (CAST) in the USA (*1*).

Ochratoxin A in Wine and Grape Juice

Occurrence

The subject of mycotoxins in fruit products such as wine and fruit juices has been reviewed (*2-4*). OTA in wine has been an important topic of concern to food regulatory authorities (*5- 8*).

The main fungus associated with presence of OTA in grapes and responsible for its occurrence in wine musts is the black aspergillus *Aspergillus carbonarius* (*9-12*). A related ochratoxigenic species *A. tubigensis* can also contaminate grapes (*13*).

OTA was first found in wine and grape juice in Switzerland by Zimmerli and Dick (*14*) just over 10 years ago. Since then there have been numerous reports on its occurrence in wine (Table I) with fewer reports of occurrence in grape juice (Table II). An analysis of published European findings by Bellí *et al.* (*6*) demonstrated that the mean incidence of OTA positive samples in red wines was 71%, 66% in rosé wines and in white wines 45%. A similar trend was observed for mean OTA concentrations. These differences were first noted by Majerus and Otteneder (*38*). The difference in normal distributions of OTA concentrations in red and white wines from the Mediterranean region was represented graphically in a Canadian study (*31*).

According to the European Commission Reports on Tasks for Scientific Cooperation (SCOOP), the mean level of OTA found in European wine was 0.36 ppb (*24*), while for grape juice it was 0.56 ppb (*24*). The highest levels reported worldwide were 15.6 ppb in a red Italian wine (*24*) and in a Moroccan wine (*35*) and 15.3 ppb in a Spanish dessert wine (*36*). The maximum level of OTA determined in grape juice was 6.7 ppb in a German sample (*37*). In Turkey, a grape juice produced from moldy grapes is concentrated to a syrup called pekmez, which can contain as much as 50 ppb of OTA (*34*).

Table I. Ochratoxin A in Wine Originating from Various Countries

Continent	*Countries*
Europe	Austria, Belgium (*15*), Bulgaria, Croatia (*16*), Cyprus (*17*), Czech Republic (*18*), France, Germany, Greece, Hungary (*19*), Italy, Lebanon (*20*), Macedonia, Portugal (*21*, *22*), Romania, Slovenia (*23*), Spain, Switzerland, United Kingdom (*24*),Yugoslavia
Asia	Japan (*25*), Taiwan (*26*), Turkey (*27*)
South America	Argentina (*28*), Brazil (*28*), Chile (*28*)
North America	Canada, USA (*29*)
Africa	Algeria, Morocco, South Africa, Tunisia
Australasia	Australia (*30*), New Zealand

NOTE: References for most occurrences are cited in (*31*); where this is not the case other references are indicated and some more recent references are also added.

Table II. Ochratoxin A in Grape Juice Originating from Various Countries

Continent	*Countries*
Europe	France, Germany, Italy, Norway (*24*), Poland (*32*), Spain, Switzerland, United Kingdom (*24)*
Asia	Japan (*33*), Turkey (*34*)
South America	Brazil (*28*)
North America	Canada, USA

NOTE: References for most occurrences are cited in (31); where this is not the case other references are indicated and some more recent references are also added.

Levels of OTA in red wines from the south of Europe are generally greater than in more northerly European wines (*31, 38-40*). This appears to be due to climate rather than wine making differences. Concentration increases of OTA in wines from central and southwest France towards the Mediterranean (*41*), from northern to southern Greece or the Greek islands (*42, 43*) and from northern to southern Italy (*44*) have also been observed. Studies on Carignan grape musts in Europe indicate that the OTA concentrations increase the closer the vineyard to the Mediterranean Sea (*45*). The level of OTA was found to be stable in wine for at least a year (*46*).

OTA has rarely been detected in other fruit juices. It was found in grapefruit juice analysed in Morocco (*47*) but in Switzerland the chromatographic peak seen on analysis of grapefruit juice was regarded as an interference (*14*). Very low levels of OTA (up to 0.06 ppb) were reported in some samples of black currant and tomato juices in Germany (*41*) and in fruit drinks in Poland (*32*).

Methods of Analysis for Ochratoxin A in Wine

OTA in wine is usually determined by reversed phase liquid chromatography (LC) with fluorescence (*48-53*) or mass spectrometric detection (*54-58*). Limits of OTA quantitation using fluorescence are in the range 0.02-0.08 ppb. Automation has been applied (*50*, *58*). Cleanup of the wine by an immunoaffinity column is used in most cases and diluted wine may be applied directly to the immunoaffinity column, as in the Official Method of AOAC International (*49*). Gas chromatography with mass spectrometric detection was evaluated by Soleas *et al.* (*59*) as a confirmatory system but it was not recommended for quantitation of OTA in wine. Castellari *et al.* (*60*) compared different immunoaffinity column cleanup procedures while Leitner *et al.* (*54*) compared C_{18} and immunoaffinity column methods. C_{18} solid phase extraction seems to be a suitable alternative to immunoaffinity column cleanup (*52*, *57*, *61*). Other types of solid phase extraction columns, including anion exchange/reversed phase, phenylsilane and polymeric based columns have also been investigated (*52*, *56*, *62*). Liquid phase micro-extraction using 1-octanol immobilized in the pores of a porous hollow fiber (*63*) and solid phase micro-extraction where the fiber is simply immersed in diluted wine (*64*) have been investigated for isolation of OTA from wine; the latter technique gave with reversed phase LC and fluorescence a limit of detection of 0.07 ppb. Two attempts have been made to use a molecularly imprinted polymer to selectively bind OTA in wine analysis. One study found that an imprinted polymer performed the same as a non-imprinted polymer, indicating that the imprinted binding sites played a minor role in selective OTA retention (*65*); the other utilized molecularly imprinted polypyrrole modified carbon nanotubes to give an LC detection limit of 0.012 ppb (*66*). Dall'Asta *et al.* (*67*) avoided cleanup altogether and directly injected wine into the LC system, still obtaining a detection limit by fluorescence of 0.05 ppb. Immunochemical methods did not achieve the same level of sensitivity as LC methods. An electrochemical immunosensor (*68*) and a membrane dot immunoassay (*69*) gave detection limits of 0.9 ppb and 1 ppb, respectively, but an array biosensor was not sensitive (detection limit 38 ppb) (*70*) and would not be suitable for enforcement of the EU regulations, which have a maximum limit for OTA of 2.0 ppb in wine, grape juice and grape must (*71*).

Ochratoxin A in Wine: Prevention and Treatment

In view of the widespread occurrence of OTA in wine, considerable effort has been made in recent years that aimed to prevent its formation in grapes (vineyard management), to study its fate in the winemaking process and carry out research on treatment of wine and grape juice to remove OTA.

Certain fungicides can stop growth of *Aspergillus carbonarius* on grapes, which together with complementary protection against the grape berry moth results in lower levels of OTA in the musts (*72-74*).

In the processes of crushing and maceration, OTA levels increase in the must because of extraction from the grapes but in the subsequent alcoholic fermentation, racking and malo-lactic fermentation of the wine, decreases in OTA concentrations occur (*75*). However, poor winery sanitation may result in contamination of the equipment with molds which can contribute to OTA in the must (*76*). The main losses of OTA occur in the solid–liquid separation after fermentation (*77*, *78*). Overall losses of more than 90% of OTA initially present in the grapes have been reported in red and white wine ready for consumption (*78*). Removal of OTA during fermentation was dependent on the yeast strain (*79*). In a study where the must was spiked before vinification to port wine, the levels of OTA dropped by up to 92% (*80*). Radiolabeling of OTA with tritium and analysis by radio-LC of the filtrate and solid after fermentation indicated that OTA did not appear to be transformed to other products by the yeast (*81*).

Fining (clarification) is a common winery practice. Potassium caseinate and activated charcoal were found experimentally to be the best fining agents to remove OTA from wine (*82*). Other effective materials were silica gel, gelatin, and bentonite (*76*, *82*, *83*). Microbiological adsorbents of OTA from wine or grape juice are active dry yeast (*84*), yeast lees (*84*), heat treated yeast (*Saccharomyces cerevisiae*) (*85*), *Lactobacillus plantarum* (*86*), and living and dead conidia of black Aspergilli (*87*, *88*).

Other Mycotoxins in Wine

Ochratoxin C

The toxic ethyl ester of OTA, ochratoxin C, has been found in wine at about 10% (range 5-25%) of the level of OTA (*39*), probably formed from OTA and ethanol in the acidic environment.

Alternaria Toxins

Alternaria spp. frequently contaminate many kinds of fruits, including grapes (*89*, *90*). Recently, Canadian and imported red and white wines were analyzed for two of the toxins of *A. alternata*, alternariol (AOH) and alternariol monomethyl ether (AME) (*90, 91*). As determined by LC-MS/MS, AOH was found in 13 out of 17 Canadian red wines at levels of 0.03 to 5.02 ppb and in 7 out of 7 imported red wines at 0.27-19.4 ppb, usually accompanied by lower concentrations of AME (up to 0.2 ppb in Canadian red wine) (*90*).

Mycophenolic Acid

The *Penicillium* mycotoxin mycophenolic acid was found in 91% of red wine samples analyzed in Germany (*92*). The maximum concentration found was 130 ppb and samples from southern Europe (Greece and Spain) were the most contaminated. Another *Penicillium* mycotoxin, citrinin, was not detectable in any sample.

Trichothecin and Related Metabolites

Trichothecium roseum occurs on grapes in Germany and its metabolites trichothecin (up to 500 ppb) and the related compounds trichothecolone and rosenonolactone have been detected in grapes and wines in that country (*93*, *94*). Trichothecin is cytotoxic (*93*). The mycotoxin containing wines were Auslese, Trockenbeerenauslese and Muscatel. Trichothecolone, rosenonolactone and iso-trichothecin, in addition to two related metabolites, were formed from trichothecin during the fermentation of grape juice (*95*).

Aflatoxins

Several surveys from 1967-1977 reported the presence of aflatoxins in wine (*96-99*). Two of 33 German white wines contained less than 1 ppb aflatoxin B_1 as determined by thin layer chromatography (TLC) (96) but identity was not confirmed by an additional test. Another TLC study found aflatoxins B_1, B_2, G_1 and G_2 in 5 out of 22 European wine samples (from 1.2 to 2.6 ppb total aflatoxins in three red wines and two dessert wines); confirmation of identity was by sulfuric acid spray (*97*). Eleven other red and dessert wines appeared to contain traces of aflatoxins (< 1 ppb). An unpublished report (*98*), again using TLC, but with identification in 9 solvent systems, indicated the presence of aflatoxins B_1, B_2, G_1 and G_2 in 33 samples of mainly European red, white and dessert wines (up to, respectively, 4.1, 14 and 49 ppb total aflatoxins B_1, B_2, G_1 and G_2). In a U.S. survey, the hemiacetal aflatoxin B_{2a} was detected by LC in a French Sauterne wine (0.3 ppb) and in a plum wine (0.05 ppb) (*99*). The subject of aflatoxin occurrence in wines should be revisited using modern analytical methods. The hemiacetals aflatoxins B_{2a} and G_{2a} could result from the acidity and ethanol content of wine but are not regarded as very toxic.

Other Mycotoxins in Fruit Juices

Patulin

Patulin is one of the most important mycotoxins occurring in fruit juices (*1-3*). Its chemical properties, toxicity, methods of analysis, occurrence and control have been reviewed (*100*, *101*). Originating from *Penicillium expansum* growing on apples, patulin occurs frequently in apple juice as shown in surveys from many countries, including Australia, Austria, Belgium, Brazil, Canada, France, Germany, Italy, Japan, New Zealand, Taiwan, Turkey, South Africa, USA and the United Kingdom. The highest level ever determined was 45 000 ppb in freshly pressed apple cider in the USA (*100*). Patulin has also been found in other fruit juices, including grape, pear, peach, cherry, raspberry, mulberry, strawberry, pineapple, sour cherry, blackcurrant and orange juices (*1*, *101-105*). The commonly used methods of analysis use extraction of juice with ethyl acetate, cleanup by extraction with aqueous sodium carbonate and reversed phase LC with UV detection at 276 nm; cloudy apple juices are pretreated with pectinase to clarify them (*100*, *101*). Solid phase extraction techniques have also been used for cleanup. No enzyme-linked immunosorbent assay (ELISA) has been developed for detection of patulin.

The regulatory limit for patulin in the EU is 50 ppb in fruit juices and fruit nectar, in particular apple juice, and 10 ppb in apple juice and solid apple products for infants and young children (*106*). The 50 ppb limit for apple juice has been adopted by the Codex Alimentarius Commission, while a 50 ppb limit for fruit juices in general has been adopted by some countries (*107*).

It is well known that the yeast *Saccharomyces cerevisiae* greatly reduces patulin levels during alcoholic fermentation of apple juice (*108-110*), with formation of E- and Z-ascladiol (*110*). Surprisingly then, patulin has been found in French commercial alcoholic apple ciders (*111*). In line with the fermentation losses in apple juice, patulin was not detected in wine made from patulin-containing grape juice (*112*).

Storage of apple juice concentrates for one month at 22 °C reduced patulin levels by 45-64%, and after four months it was not detectable (*113*). There was greater reduction of patulin in both apple juice and a juice-like model system in the presence of L-ascorbic acid (*114*, *115*). On the other hand, patulin was stable in grape juice (and wine) for 1 month at room temperature (*116*). Various methods have been investigated for reduction of patulin in apple juice: sorting of infected apples before processing, addition of ascorbic acid, sulphur dioxide or charcoal (which is not feasible on a commercial scale), fermentation, irradiation, pressing followed by filtration, enzyme treatment and fining with bentonite (*100*, *101*, *117*). Efficiency of these methods varied.

Alternaria Toxins

In contrast to wine, red grape juices contained only sub-ppb levels of alternariol (AOH) or alternariol monomethyl ether (AME), except for one sample (of U.S. origin) which contained 39 ppb AME (*90*). White wines, white grape juices and cranberry juices contained little or no AOH/AME. AOH (≤5.5 ppb) and AME have also been found in apple juice, apple juice concentrates, raspberry juice and prune nectar (*89*, *91*). Further monitoring of levels of occurrence and toxicological studies on *Alternaria* toxins are desirable.

Miscellaneous Mycotoxins in Fruit Juices

The *Trichothecium* mycotoxin trichothecin, as might be expected from its presence in wine, has also been found in grape must in Germany (at levels up to 21 130 ppb) (*93*). There appears to have been no further investigation on this subject since 1994 (*95*).

Citrinin was detected in single samples of apple, tomato juice, cherry and black currant juices in Germany (*118*) and aflatoxin B_1 in 2 out of 5 samples of guava juice and aflatoxins B_1 and G_1 in 5 out of 5 apple beverages in Egypt (*119*).

Mycotoxins in Beer

Ochratoxin A in Beer

As is the case with OTA in wine, considerable research and monitoring of OTA in beer has been carried out since the subject of mycotoxins in beer was reviewed in 1996 (*120*). A pilot scale study on making beer from contaminated malting barley showed that 13-32% of the OTA initially present in the barley survived the overall brewing process, with substantial losses (up to 40%) observed in the mashing process (121). Countries where OTA has been found in beer are listed in Table III. The highest level ever reported - 110 ppb in a European beer - was in fact the first finding of natural occurrence of OTA in beer (*139*); the analyses were performed by TLC but without confirmation. Apart from this study, the highest levels that have been found worldwide were 8.1 ppb (in Turkey) (*137*) and 0.29 ppb in the EU SCOOP report (*24*). In general OTA concentrations in positive samples are low: the mean level in European beers according to the SCOOP report was 0.03 ppb (n=496) (*24*) and the mean level found in Belgian beers was 0.033 ppb (n=60) (*123*). No maximum level for OTA in beer has been established by the EU (*140*). Instead a maximum level for OTA of 3.0 ppb in malt was set. The Netherlands, Finland and Italy have guidance levels of 0.5, 0.3 and 0.2 ppb, respectively, for OTA in beer (*141*).

Method development for OTA in beer has actively paralleled that for wine (*62*). The AOAC International Official method employs immunoaffinity column

Table III. Ochratoxin A in Beer Originating from Various Countries

Continent / Region	*Countries*
Europe	Austria (*122*), Belgium (*122-127*), Czech Republic (*128*), Denmark (*24*, *122*, *123*, *125-127*, *129*), Finland (*24*), France (*122*, *123*, *126*), Germany (*24*, *120*, *122*, *123*, *125-127*, *130*, *131*), Hungary (*19*) , Ireland (*122*, *127*), Italy (*24*, *122*, *123*, *126*, *127*), the Netherlands (*122*, *123*, *125*, *126*), Portugal (*21*, *24*, *122*), Spain (*24*, *122*, *125*, *126*, *132*, *133*), Sweden (*24*), Switzerland (*14*), United Kingdom (*122*, *123*, *126*, *127*)
Asia	China (*122*), India (*122*), Indonesia (*122*), Israel (*122*), Japan (*25*, *35*, *122*, *134*), Korea (*135*) , Lebanon (*136*), Philippines (*122*), Singapore (*122*), Taiwan (*26*, *122*), Thailand (*122*), Turkey (*137*), Vietnam (*122*)
South America	Bolivia (*122*), Brazil (*122*,*138*)
North and Central America	Canada (*122*), Jamaica (*122*), Mexico (*122*, *125*, *126*), Trinidad (*122*), USA (*122*)
Africa	Kenya (*122*)
Australasia and Pacific	New Zealand (*122*), Tahiti (*122*)

cleanup of beer and reversed phase LC with fluorescence detection (*49*). The beer sample is diluted with polyethylene glycol and sodium bicarbonate solution prior to cleanup. A C-18 or other SPE column can also be used for cleanup of degassed beer (*59*, *62*) and instead of fluorescence detection, a photodiode array detector (*59*) or MS/MS detector can be applied (*142*). Solid phase micro-extraction, where the fiber is immersed in degassed beer (*141*), has been investigated for isolation of OTA from beer (cf., wine); this technique gave a limit of quantitation with reversed phase LC and fluorescence of 0.053 ppb. The determination of OTA in foods in general has been reviewed (*143*).

Fusarium Toxins in Beer

The occurrence of *Fusarium* toxins in beer is summarized in Table IV. Most information concerns deoxynivalenol (DON). DON can originate in the malt or grain adjuncts used in the process of making beer. As it is stable to fermentation and to the boiling of wort, there can be considerable carryover to finished beer (*120*). Of interest is the high incidence of nivalenol in Korean beers (*147*) which suggests that additional surveys for its presence should be carried out in those countries where this mycotoxin occurs in the grains in significant concentrations. The other important mycotoxins found in beer are the carcinogenic fumonisins B_1 and B_2, which are probably introduced into the beer from contaminated corn-based brewing adjuncts. The highest level determined was 85.5 ppb in a Spanish beer, analysed by ELISA (*155*). High incidences of contamination with fumonisins - 43% in Spanish beers (detection limit 3 ppb) (*155*) and 84% in US beers (detection limit 0.3 ppb) (*156*) - have been reported.

Table IV. *Fusarium* Toxins Found in Beer

Mycotoxin	*Country /Region*	*Incidence*	*Max. Level (ppb)*	*Reference*
Deoxynivalenol	Canada	21/33	50	*120*
	Europe	(e.g. 272/313) 22/50	57	*144*
	Argentina	7/72	221	*145*
	Brazil	8/36	336	*146*
	Korea	17/17	5.3	*147*
	Japan	75/75	1.4	*148*
	Kenya		6.4	*149*
Nivalenol	Canada	1/25	0.12	*150*
	Korea	33/36	38	*147*
T-2 toxin	Brazil	3/72	249	*146*
Zearalenone	Kenya	75/75	0.01	*149*
	Other African countries	83/278	53 000	*120, 151*
	USA	1 sample	0.26	*152*
Fumonisin B_1	Canada	8/31	15	*153*
	Canada and imports	22/46	53	*154*
	Spain	14/32	86	*155*
	USA	21/25	13.5	*156*
	Mexico	3/3	<1	*156*
	Slovakia	0/20	< 0.7	*157*
	Kenya	54/75	0.78	*149*
	South Africa (maize beer)	18/18	1066	*158*

Aflatoxins in Beer

Aflatoxins have been detected as natural contaminants of barley, maize and sorghum malts (*120*). Up to 20% of the amount of aflatoxin B_1 added to malt or corn grits can survive the brewing of beer. With the use of sensitive methods, including immunoaffinity column cleanup for LC determination, the presence of parts per trillion (ppt) concentrations of aflatoxins B_1 and B_2 has been demonstrated in commercial beer from many parts of the world, notably in warm countries such as India (up to 230 ppt aflatoxin B_1), Japan, Philippines, Mexico, Brazil, Peru, and Bolivia (*120*, *122*, *159*, *160*). Aflatoxins have been detected in only a few samples of European beers (from Spain and Portugal) using methods where detection limits ranged down to 0.7 ppt of aflatoxin B_1 (*120*, *122*, *160*). Up to 1.5 ppt aflatoxin B_1 has been found in U.S. and Canadian beers at very low incidence (*122*, *160*). Generally, with the exception of a negative survey on 75 bottled Kenyan lager beers (*149*), much higher levels of aflatoxins have been found in African beers - both commercial and home-brewed (*120*, *161*, *162*).

Conclusions

This overview of mycotoxins in alcoholic beverages and fruit juices has mainly focused on their occurrence and on methods for their analysis and should provide a starting point for further research and surveillance. Ochratoxin A, aflatoxins and fumonisins are known carcinogens so merit attention. Some mycotoxins previously found in wine need further investigation, namely aflatoxins, mycophenolic acid, trichothecin, related *Trichothecium* metabolites, and the *Alternaria* toxins alternariol and alternariol monomethyl ether. Analysis of fruit juices, particularly grape juice, for these mycotoxins should accompany these investigations. Wine should be re-analysed for aflatoxins B_1, B_2, G_1 and G_2 using modern instrumentation. In addition to continued research on prevention and removal of OTA in wine, the toxic ethyl ester of OTA, ochratoxin C, should be looked for.

The significance of finding fumonisins in beer needs to be evaluated and further surveillance carried out. While the trichothecene DON is well known to occur in beer, additional surveys for presence of the related and more toxic mycotoxin nivalenol should be carried out in those countries and regions where it occurs in the grains in significant concentrations.

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Chapter 10

Recent Developments in Trichothecene Analysis

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Trichothecenes are a group of toxic metabolites mainly produced by several *Fusarium* species occurring in cereals. Gas-chromatographic methods (GC-ECD or GC-MS) are widely used for quantitative determination of the more toxic type-A trichothecenes, i.e., T-2 and HT-2 toxins. Deoxynivalenol (DON), the main type-B trichothecene frequently occurring in wheat, is commonly detected by liquid chromatography (LC)-UV with good accuracy and precision. Recently, at ISPA-CNR a new method has been developed for the simultaneous determination of T-2 and HT-2 toxins in cereal grains at ppb levels using immunoaffinity column clean-up, labeling with 1-anthroylnitrile, and LC with fluorescence detection (FD). Moreover a fluorescence polarization (FP) immunoassay has been developed at USDA-ARS-NCAUR for rapid quantification of deoxynivalenol (DON) in wheat. Results of recent researches on the improvement of methods for the determination of T-2 and HT-2 toxins in cereals by LC-FD and new labeling reagents (i.e., 1-naphthoyl chloride, 2-naphthoyl chloride, pyrene-1-carbonyl cyanide) and by capillary electrophoresis-laser induced fluorescence (CE-LIF) detection are presented, together with the optimization of the FP immunoassay for rapid screening of DON in common wheat, durum wheat, semolina, and pasta.

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Introduction

Trichothecenes are a group of secondary metabolites produced by several fungal genera including *Fusarium, Trichoderma, Cephalosporium, Myrothecium, Trichothecium* and *Stachybotrys* (*1*).

Trichothecenes produced by *Fusarium* species belong to two main categories: 'type-A' which are characterized by a functional group other than a carbonyl at C-8, and 'type-B' which have a carbonyl group at C-8. T-2 toxin and HT-2 toxin are type-A trichothecenes, while deoxynivalenol (DON), also known as vomitoxin, is a type-B trichothecene. These toxins are commonly found in cereals including wheat, barley, maize, oats, rye, and derived products, particularly in Europe and in cold and wet climatic regions. A recent survey on the occurrence of *Fusarium* toxins in food in the European Union (EU), in order to assess the dietary intake by the population of EU member states, showed an incidence of positive samples of 57% out of 11,022 samples analyzed for DON and 20% and 14% out of 3,490 and 3,032 samples analyzed for T-2 and HT-2 toxins, respectively. Wheat and maize were the cereals most frequently contaminated by DON; maize, wheat and oats by T-2 and HT-2 toxins (*2*). Similar results were reported on globally by the Joint FAO/WHO Expert Committee on Food Additives (JECFA) in the fifty-sixth report, which was on the safety evaluation of mycotoxins in food (*3, 4*).

Toxicological studies have shown that T-2 toxin, HT-2 toxin and DON have strong inhibitory effects on protein, DNA, and RNA synthesis and cause toxic effects on cell membranes. In addition, T-2 toxin, which is considered the most toxic trichothecene, has immunosuppressive and cytotoxic effects, and has been shown to cause hemorragic diseases and necrosis and inflammation of the skin and mucosal surfaces. The little direct information on the toxicity of HT-2 toxin which is available indicates that it induces adverse effects similar to T-2 toxin. DON has also been shown to cause vomiting, haematic, and anorexic syndromes as well as neurotoxic and immunotoxic effects in mammals (*3-5*).

Due to their toxic effects, the contamination of cereals and cereal-based products by trichothecene mycotoxins represents a real risk for human and animal health. In order to protect human health from exposure to these mycotoxins through the consumption of cereal-based food products, several countries have established regulatory or guideline limits in raw materials and foods intended for human consumption. In particular, a few dozen countries have set limits for DON, whereas Armenia, Belarus, Bulgaria, Estonia, Hungary, Latvia, Moldova, the Russian Federation, Slovakia, and Ukraine have fixed limits for T-2 toxin (*6*). The US Food and Drug Administration (FDA) has issued an advisory level of 1.0 µg/g for DON in milled wheat products (e.g., flour, bran, and germ) that may be consumed by humans (*7*). Harmonized regulations for DON in food, including official protocols for sampling and analysis, were implemented on July 1st 2006 within the European Union, whereas permissible levels of T-2 and HT-2 toxins in cereal-based products are currently under discussion (*8, 9*).

Analytical methods for rapid, sensitive, and accurate determination of these mycotoxins in cereals and cereal-based products are required in order to protect consumers from the risk of exposure, to allow monitoring programs (rapid screening of materials in the food/feed production chain) and to ensure that regulatory levels fixed by the EU or other international organizations are met. Gas chromatographic (GC) methods based on electron-capture (ECD), flame ionization (FID), and mass spectrometric (MS) detection are the most widely used methods for quantitative determination of T-2 and HT-2 toxins. DON is commonly measured at ppb levels by high performance liquid chromatographic (LC) methods based on ultraviolet (UV) and MS detection. These methods require preliminary clean-up of extracts in order to obtain good sensitivity, are time-consuming, expensive, and unsuitable for screening purposes. Enzyme-linked immunosorbent assay (ELISA) methods using polyclonal or monoclonal antibodies have been developed for rapid screening of trichothecenes in cereals. However, ELISA methods show strong cross-reactivity against mycotoxin analogues (i.e., acetylated-DON), involve multiple washing steps, and require long incubation times for complete antigen-antibody reaction *(10, 11)*. Recently, a variety of emerging immunoassays have been proposed for the rapid analysis of DON or T-2 toxin in several matrices. They are based on novel technologies including lateral flow devices (LFD), membrane-based flow-through enzyme immunoassay, fluorescence polarization (FP) immunoassay, and surface plasmon resonance (SPR) biosensors (*12-14*).

This paper summarizes results of some of the recent research carried out at the Institute of Sciences of Food Production of the National Research Council (ISPA-CNR, Bari, Italy) and at the Mycotoxin Research Unit of the USDA-ARS-NCAUR (Peoria, IL, USA) aimed at improving determination of T-2 toxin, HT-2 toxin, and DON in cereals and cereal-based products.

LC with Fluorescence Detection for T-2 and HT-2 Toxins

Intake estimates indicate clearly that the presence of mycotoxins in the diet at low levels can be of some concern for public health. Recently, the Joint FAO/WHO Expert Committee on Food Additives (JECFA) and the Scientific Committee on Food (SCF) of the European Commision pointed out that data on the presence of T-2 and HT-2 toxins in food are limited and of low quality and they should not be used for intake estimations. In addition, the sensitivity of the analytical methods used was poor in many cases which can lead to an overestimation of the intake. Therefore the development of a sensitive method for T-2 and HT-2 toxins and collection of more data on their presence in cereal and cereal-based products, in particular in oats and oat products, are of high priority for reliable dietary exposure assessments (*4, 8*).

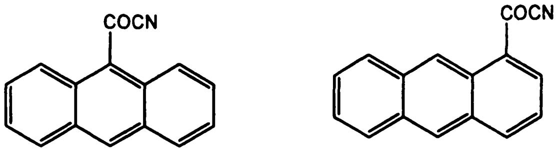
Several chromatographic (TLC, GC-ECD, GC-FID, GC-MS and LC-MS) and enzyme-linked immunoassay (ELISA) methods have been developed for the determination of T-2 toxin, alone or in combination with other trichothecenes (*10, 15*). At present, GC with ECD or MS detection are the techniques most used for quantification of type-A trichothecenes, although a pre-column derivatization step of the extracts is necessary to increase volatility of the toxins and provide higher sensitivity. Recently, a comparative collaborative study on method performance for analysis of trichothecenes (including T-2 and HT-2 toxins) by GC methods clearly showed that method improvements are needed with respect to recovery, accuracy, and precision of the measurements. The main problems are derived from matrix interferences that induced enhancement of the trichothecene response, with consequent overestimation of the contamination levels (*16*).

LC with fluorescence detection (FD) gives high sensitivity, selectivity, and repeatability of measurements, but is not applicable to trichothecene detection due to the lack of appropriate chromophores in their structure. Although specific labeling reagents are commercially available for hydroxyl groups to form fluorescent esters, their use in trichothecene analysis showed low reaction yield or interfering peaks in the chromatogram.

A simple procedure for the simultaneous determination of T-2 toxin, HT-2 toxin, T-2 triol, and T-2 tetraol by LC-FD has been developed (*17*). It involved the synthesis of the labeling reagent, coumarin-3-carbonyl chloride, an optimized derivatization reaction and a procedure for clean-up of the reaction mixture to remove excess reagents and by-products. Recently, the derivatizing reaction with coumarin-3-carbonyl chloride was used for developing a method for the determination of T-2, HT-2 toxin, neosolaniol, and diacetoxyscirpenol in cereal cultures of *Fusarium sporotrichioides* on maize, rice, and wheat by LC-FD after solid phase extraction (SPE) column clean-up. Although the method had good sensitivity, its applicability to cereal samples showed low toxin recoveries (*18, 19*).

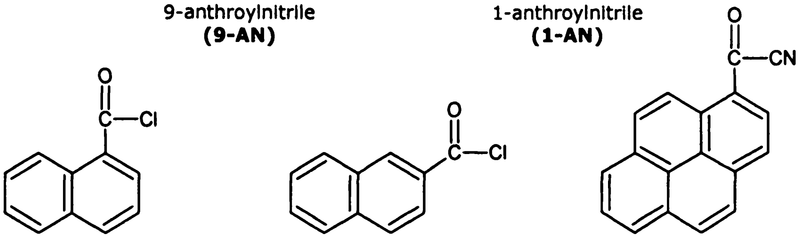
Two commercially available fluorescent reagents for modifying alcohols, 9-anthroylnitrile (9-AN) and 1-anthroylnitrile (1-AN) (Figure 1), were tested for labeling T-2 toxin in order to make it detectable by LC-FD. Due to its high efficiency for a variety of acylation reactions, the base 4-dimethylaminopyridine (DMAP) was used as catalyst for the reaction. The derivatization conditions were optimized by investigating different reaction solvents, reagent molar ratios, temperatures, and reaction times. Both 1-AN and 9-AN reagents reacted with the hydroxyl group of T-2 toxin under mild conditions to form the corresponding esters. The T-2-(1-AN) derivative gave up to 15 times higher fluorescence intensity than the T-2-(9-AN) derivative in all experiments performed. Experiments to test the stability of T-2 anthroyl ester showed no decrease of intensity of fluorescence of T-2 derivative up to 5 days for solutions stored in the dark and under light. The derivatizing reaction has been used to develop a sensitive, reproducible, and accurate method for the determination of T-2 toxin in wheat, corn, barley, oats, rice, and sorghum (*20*). The method uses immunoaffinity columns (IACs) specific for T-2 toxin for clean-up of cereal extracts, pre-column derivatization with 1-AN, and LC-FD for T-2 toxin determination. Recoveries from different cereals spiked with the mycotoxin at levels ranging from 50 μg/kg to 1,500 μg/kg were from 80% to 99%, with within-laboratory relative standard deviation (RSD_r) lower than 6% for all spiking levels. The limit of detection was 5 μg/kg, based on a signal-to-noise-ratio of 3:1. The use of IACs allowed rapid clean-up and provided clean extracts to be analyzed by LC due to the specificity of the antibody. The method allowed the determination of T-2 toxin at ppb (µg/kg) levels in various cereals with good accuracy and precision, enabling measurement of the toxin at levels that can occur in naturally contaminated cereal samples (*20*).

By applying the same derivatization procedure, a stable fluorescent derivative was obtained also with HT-2 toxin (*15*). In order to develop a method based on LC-FD and IAC clean-up for the simultaneous determination of T-2 and HT-2 toxins in cereal grains, the specificity of the anti-T-2 toxin antibody was evaluated against different structurally related trichothecene mycotoxins: deoxynivalenol (DON), nivalenol (NIV), T-2 toxin, T-2 triol, T-2 tetraol, HT-2 toxin, and acetyl T-2 toxin. The antibody cross-reacted 100% with T-2 and HT-2 toxins, and 90% with T-2 triol and acetyl T-2 toxin. The high cross-reactivity of the antibody with T-2 triol and acetyl T-2 should not be a problem for the LC determination of T-2 and HT-2 toxins after pre-column derivatization with 1-AN. In particular, acetyl T-2, which lacks free hydroxyl groups, should not react with 1-AN, while T-2 triol, carrying three hydroxyl groups, should react with 1-AN to form derivatives with polarity quite different from (and therefore not interfering with) the T-2 and HT-2 anthroylnitrile derivatives. Derivatization with 1-AN and IAC clean-up were successfully applied to the analysis of T-2 and HT-2 toxins in wheat, maize, and barley. Recoveries from spiked samples with toxin levels from 25 to 500 µg/kg ranged from 70 to 100%, with RSD_r lower than 8%. The limit of detection of the method was 5 µg/kg for T-2 toxin and 3 µg/kg for HT-2 toxin, based on a signal-to-noise ratio of 3:1. The analytical method did not allow the determination of HT-2 toxin in oats because of interfering chromatographic peaks occurring at the retention time of HT-2-(1-AN) derivative (*15*). The method was also applied to the determination of T-2 and HT-2 toxins in eggs (*21*).



9-anthroylnitrile (9-AN)

1-anthroylnitrile (1-AN)



1-naphthoyl chloride (1-NC)

2-naphthoyl chloride (2-NC)

pyrene 1-carbonyl cyanide (PCC)

Figure 1. Fluorescence labeling reagents for T-2 and HT-2 toxins (commercially available)

In order to provide an improved LC-FD method with lower detection limits (LOD) for T-2 and HT-2 toxins in foodstuffs, including oats, three different commercially available fluorescent reagents were tested (Figure 1). 1-naphthoyl chloride (1-NC), 2-naphthoyl chloride (2-NC) and pyrene-1-carbonyl cyanide (PCC) reacted with the hydroxyl groups of T-2 toxin and HT-2 toxin under mild conditions to form the corresponding esters *(22)*. Excitation and emission spectra of the fluorescent derivatives were recorded and maximum excitation and emission wavelengths were selected. A wide linear range (10-1000 ng for either T-2 or HT-2 derivatized toxin), good repeatability ($RSD_r \leq 8\%$) of the reaction, and good stability (up to 2 weeks at -20 °C and five days at room temperature) of the fluorescent derivatives were obtained. Detection limits (based on a signal-to-noise ratio of 3:1) were 10.0, 6.3 and 2.0 ng for derivatized T-2 toxin and 6.3, 2.3 and 2.8 ng for derivatized HT-2 toxin with 1-NC, 2-NC and PCC, respectively. A higher fluorescence intensity was observed for derivatives obtained by reaction of T-2 and HT-2 toxins with PCC and 2-NC, with respect to those obtained with 1-AN.

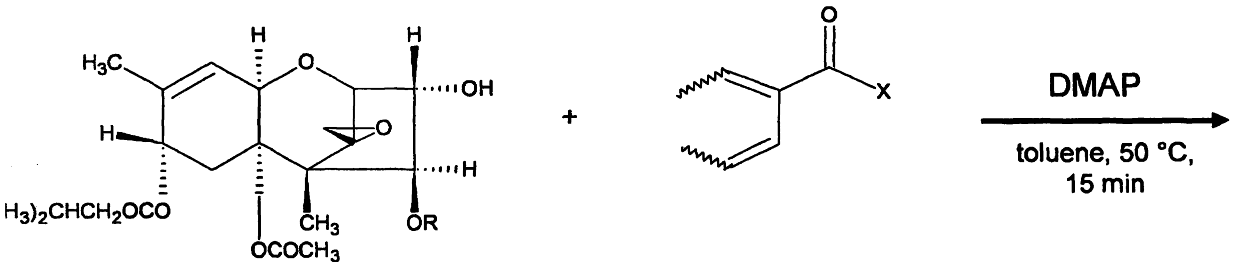
Preliminary studies showed the applicability of the new labeling reagents PCC or 2-NC for the simultaneous determination of T-2 and HT-2 toxins by LC-FD after IAC clean up in naturally contaminated cereal grains, including oats (*22*).

Under the optimized reaction conditions, with any of the tested labeling reagents, mono-derivatized products were obtained for T-2 toxin and *bis*-derivatized products were obtained for HT-2 toxin. The identity of the fluorescent derivatives was confirmed by LC-MS and LC-MS/MS (positive chemical ionization) (*15, 22*). The derivatization scheme for T-2 and HT-2 toxins with labeling reagents is reported in Figure 2.

Capillary Zone Electrophoresis with Laser-induced Fluorescence Detection for T-2 Toxin

Capillary zone electrophoresis (CZE) is an analytical technique where compounds are separated from one another based upon differences in electrical charge. This separation principle may be useful, particularly for charged mycotoxins such as the fumonisins, but can also be applied to the more hydrophobic mycotoxins such as the aflatoxins (*23, 24*). For many analytes fluorescence detection is preferred due to greater sensitivity or fewer background interferences. Recently, fluorescence-based CZE methods have been developed for determination of OTA in roasted coffee, maize, and sorghum, for the analysis of AFB_1 in maize, and for the determination of FB_1 in maize after derivatization with fluorescein isothiocynate, allowing their detection at levels commonly found in naturally contaminated food samples (*25-27*).

Because of the possibilities for increased sensitivity with the fluorescent labeling described above, T-2 toxin was derivatized and separated from the reaction mixture using CZE with laser induced fluorescence (LIF) detection. T-2 toxin was labeled with pyrene at the C-3 position after reaction with pyrene-1-


H
H
H_3C
O
OH
O
H
H
$H_3)_2CHCH_2OCO$
CH_3
OR
$OCOCH_3$
+
O
X
DMAP
toluene, 50 °C,
15 min

R = $COCH_3$ **T-2 toxin**

R = H **HT-2 toxin**

X = Cl **(1-NC, 2-NC)**

X = CN **(1-AN, 9-AN, PCC)**

In Food Contaminants; Siantar, D., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2008.

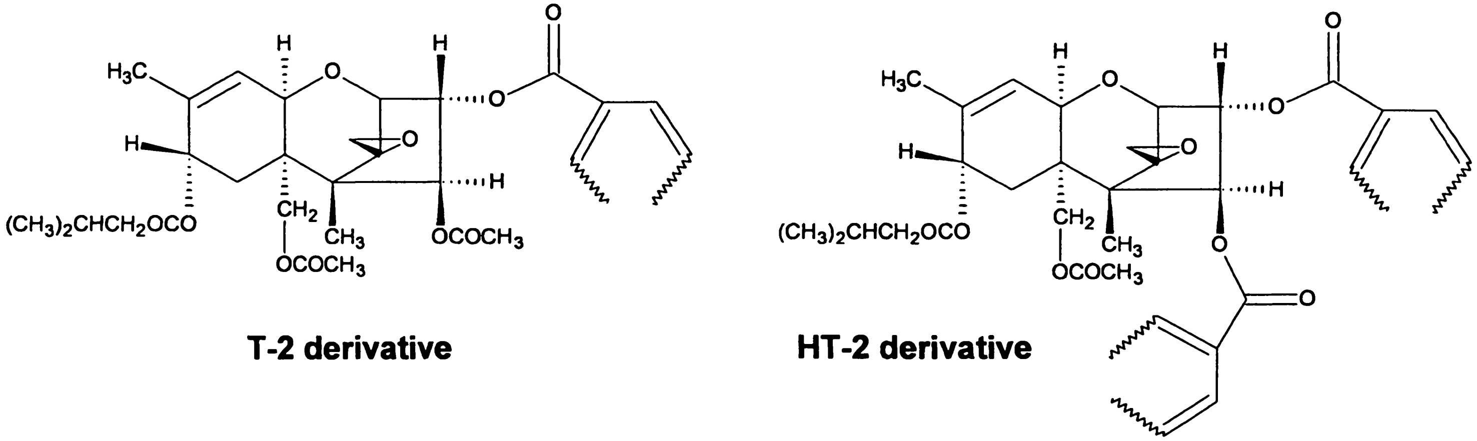


Figure 2. Scheme of the derivatization reaction of T-2 and HT-2 toxins with commercially available labeling reagents (9-AN = 9-anthroylnitrile; 1-AN = 1-anthroylnitrile; 1-NC = 1-naphthoyl chloride; 2-NC = 2-naphthoyl chloride; PCC = pyrene 1-carbonyl cyanide). From Lippolis et al., *Talanta (2007), doi:10.1016/j.talanta.2007.09.024, Copyright Elsevier B.V.*

carbonyl cyanide (PCC), rendering the toxin more hydrophobic (*22*). In order to ensure adequate solubility of the derivatized toxin, separation strategies were focused upon using buffers with high solvent strengths or lower solvent strengths in combination with detergents.

Derivatization of T-2 toxin with PCC was accomplished using conditions similar to those reported previously for the derivatization of T-2 and HT-2 toxins with 1-AN (*15*). As described above, the derivatization replaced the C-3 hydroxyl of T-2 toxin with a pyrene ester. The hydrophobicity of the derivative suggested that non-aqueous CZE, or approaches using high solvent strength or detergents, such as micellar electrokinetic capillary chromatography, would be necessary in order for the derivative to remain soluble. In fact, solubility of the derivative was an issue and for this reason a buffer containing dioxane and methanol, as well as sodium borate and sodium phosphate, was used. Figure 3 illustrates typical electropherograms obtained by using a 75 µm i.d. × 31 cm fused silica capillary at 25 °C after application of 30 KV (normal polarity) and fluorescence detection (λ_{ex} 325 nm, λ_{em} 405 nm). Under these conditions the derivatized T-2 toxin was separated from the reactants and non-specific products. While detection of the derivative was accomplished, the sensitivity of the technique was substantially poorer than LC with fluorescence detection, making LC-FD the preferred technique for measuring T-2 or HT-2 toxin in cereal grains.

Fluorescence Polarization Immunoassay for Deoxynivalenol

Fluorescence polarization (FP) immunoassay measures changes in the polarization of light caused by changes in molecular size resulting from antigen-antibody interactions. The technology has been extensively used in human and veterinary diagnostics and only recently has been reported for a number of mycotoxins including aflatoxins (AFs), fumonisins (FBs), zearalenone (ZEA), ochratoxin A (OTA), and deoxynivalenol (DON) (*28-35*).

The assay is based on the competition of a fluorescently labeled mycotoxin (tracer) with the unlabeled mycotoxin in solution for a mycotoxin-specific antibody. The technique measures the polarization value (P), defined by the equation $P = (I_V - I_H)/(I_V + I_H)$ where I_V and I_H are the intensities of fluorescence of the tracer along the vertical axis and the horizontal axis, respectively. The tracer has a low molecular weight and rotates rapidly in solution. When the antibody is added, a tracer-antibody complex is formed that slows the rate of rotation of the tracer and increases the polarization value. The presence of free mycotoxin in a contaminated extract decreases the fraction of the antibody-bound tracer and decreases the FP signal. Consequently, the polarization value P results are inversely proportional to the free unlabeled antigen (i.e., mycotoxin) content. A calibration curve with known concentrations of the mycotoxin is obtained and the unknown mycotoxin concentration is calculated from it (Figure 4).

As with other immunoassays, the selection of appropriate antibody and tracer is essential. The time required to reach the equilibrium of the antibody/

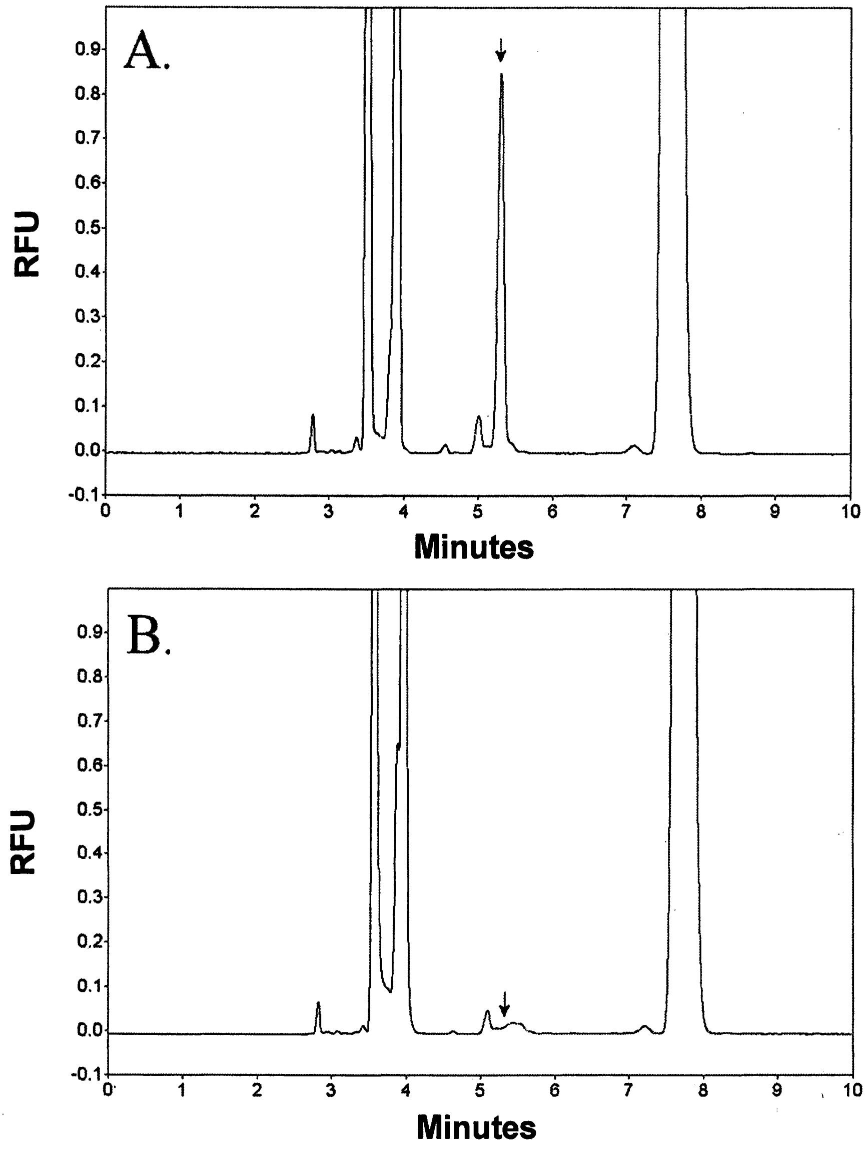


Figure 3. Electropherograms of the PCC derivative of T-2 toxin (A) or a control derivatization mixture (B). The arrow indicates the migration time of the derivatized T-2 toxin.

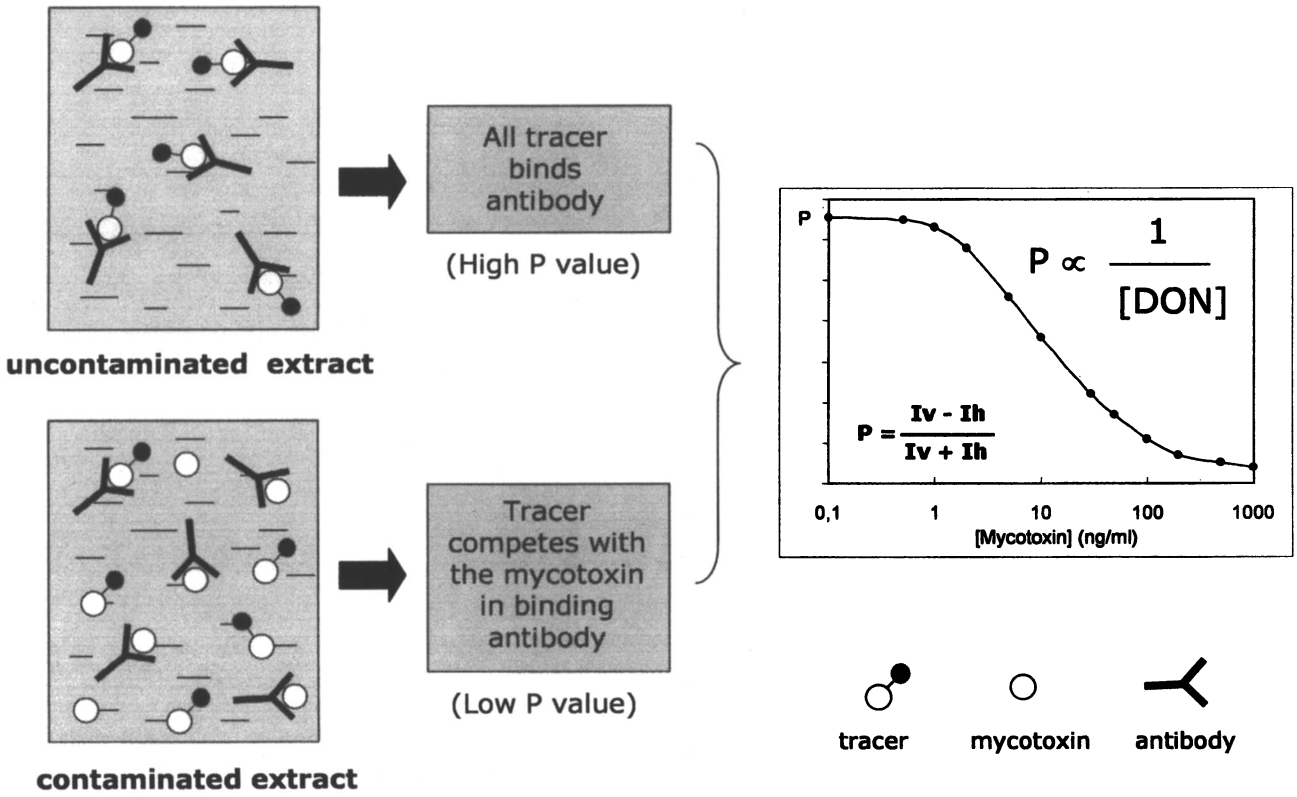

uncontaminated extract
contaminated extract
All tracer binds antibody
(High P value)
Tracer competes with the mycotoxin in binding antibody
(Low P value)
P
$P \propto \frac{1}{[DON]}$
$P = \frac{Iv - Ih}{Iv + Ih}$
0,1
1
10
100
1000
[Mycotoxin] (ng/ml)
tracer
mycotoxin
antibody

Figure 4. Principle of the FP immunoassay for the determination of mycotoxins (direct competitive immunoassay)

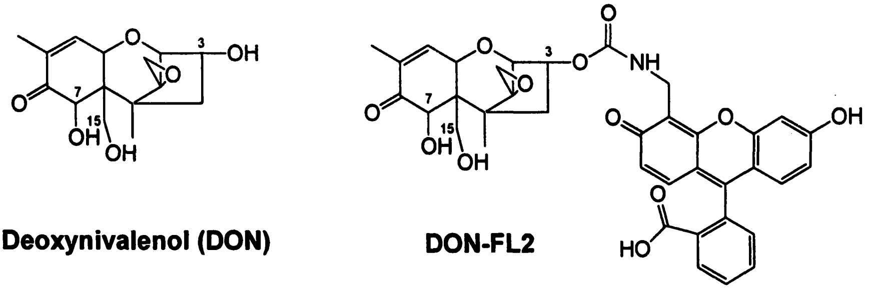
tracer/toxin interaction is a critical aspect of the FP immunoassay. This equilibration time can vary considerably, depending upon the selected antibody/tracer combination. When appropriate antibody/tracer combinations are used, rapid FP immunoassays can be developed. The FP assays have several advantages with respect to ELISA or LC methods: they are simple to perform, give highly reproducible results, and do not require clean-up steps.

Recently, three monoclonal antibodies (reference clones #1, #4, and #22) for deoxynivalenol (DON) have been developed and three DON-fluorescent tracers (DON-BODIPY, DON-FL, and DON-FL2) have been synthesized in order to develop FP immunoassays for the determination of DON in wheat (*32-34, 36*).

The first tracer (DON-BODIPY) was obtained by reaction of DON with BODIPY-8-propionic acid succinimidyl ester and used with a DON antibody in a competitive FP immunoassay. Although good results were obtained, the tracer showed a marked decomposition within a few days at low temperatures and within a day at room temperature (*32*).

The tracer obtained by reaction of DON with 6-amino-fluorescein (DON-FL) in the presence of 1,1'-carbodiimidazole showed different affinity for DON antibodies (*33*). In particular, antibodies #1 and #4 had high affinity for DON-FL yielding functional FP assays, whereas antibody #22 showed no affinity for DON-FL. Furthermore, the sensitivity of the assays was drastically affected by the length of the incubation time with the tracer. Equilibrium was reached more quickly with the antibody #1 than antibody #4. In addition, although a good correlation was observed, the FP assay showed DON overestimation in naturally contaminated wheat samples, compared with results obtained with an LC-UV method. The reason of this overestimation was unknown, but it may have been related to the high cross-reactivity of the antibody #1 with 15-acetyl DON (358% relative to DON) and the presence of this DON analogue in naturally contaminated samples (*33*).

In order to improve speed and sensitivity of the assay, a new tracer, DON-FL2 (Figure 5), was synthesized by reaction of DON with 4-aminomethyl fluorescein (*34*). The tracer reacted rapidly with antibody #22 allowing the development of a rapid FP immunoassay for DON in wheat. The method was an improvement of the previous FP immunoassay that used DON-FL as tracer and combined a rapid extraction step (3 min) with a rapid detection step (2 min). Antibodies #1 and #4 did not show relevant affinity for DON-FL2. Antibody #22 was tested against 21 trichothecenes in the FP immunoassay format. The only trichothecenes affecting the assay were DON, 3-acetyl DON (3-AcDON), and 15-acetyl DON (15-AcDON). Cross-reactivity relative to DON was 339% for 3-AcDON and 8% for 15-AcDON. The FP method was tested against spiked and naturally contaminated wheat and maize samples and compared with a widely used LC-UV method. Also in this case a systematic overestimation of DON contents was observed for both tested matrices over the entire range tested (0.5-20 μg/g), with much higher values in maize samples than in wheat samples. This indicated that the overstimation of DON content by FP immunoassay may not be merely due to the high cross-reactivity of the antibody with acetylated analogues but may have been due to matrix effects (*34*).



Deoxynivalenol (DON)

DON-FL2

Figure 5. Chemical structures of DON and DON-fluorescein tracer (DON-FL2)

The FP immunoassay using DON-FL2 tracer and antibody #22 was further investigated in order to identify the causes of the biased results and to optimize the assay for DON determination in wheat and derived products, i.e., durum wheat, common wheat, semolina, and pasta (*35*). In addition a new synthetic procedure was developed for the DON-FL2 tracer leading selectively to a product with high yields. In particular, DON-FL2 was synthesized by reaction of DON with 4'-aminomethyl-fluorescein after protection of the C7- and C15-hydroxyl groups of DON by reaction with 1-butylboronic acid to form a cyclic boronate ester (*35*).

By comparison with a widely used LC/immunoaffinity clean-up method (*37*), a FP background signal strictly depending on the amount of testing materials was observed in samples of durum wheat, common wheat, semolina, and pasta free of DON, 3-AcDON, and 15-AcDON. The same background signals were observed in spiked samples. The experiments showed that the DON amount calculated from the FP signal was directly proportional to the amount of matrix analyzed independent of the real DON content in the samples. Average values of DON overestimation were 0.27 ± 0.03 μg/g (CV = 11%) for durum wheat, 0.39 ± 0.06 μg/g (CV=15%) for common wheat, 0.08 ± 0.01 μg/g (CV = 13%) for semolina, and 0.04 ± 0.01 μg/g (CV = 25%) for pasta. The values derived from the FP analysis of 150 samples at different DON spiking levels and different amounts of analyzed matrix. These results showed that the DON overestimation was due to a matrix effect, and could not be attributed to the presence of other fungal metabolites that cross-react with the DON antibody, as previously hypothesized by Maragos and Plattner (*34*). The matrix effect for semolina and pasta was lower than that observed for durum and common wheat, possibly due to lower amounts of compounds cross-reacting with the antibody that remained in the wheat products after milling and processing.

Average recoveries determined by FP immunoassay (after subtracting the background DON level) from durum wheat, common wheat, semolina, and pasta spiked with DON at levels from 0.25 to 1.75 μg/g were 98%, 100%, 102%, and 101%, respectively, with relative standard deviations lower than 5%

(quadruplicate measurements). The limit of detection was 0.08 µg/g DON for all matrices. The overall analysis time for DON in wheat based products, including sample preparation and FP immunoassay measurement, was less than 10 min.

Comparative analyses of naturally contaminated samples performed by the FP immunoassay method (after subtracting the background DON level) and LC/immunoaffinity clean-up method showed a good correlation between DON concentrations (r > 0.9955). In addition good agreement between results was obtained after correction of the data for recoveries (Figure 6).

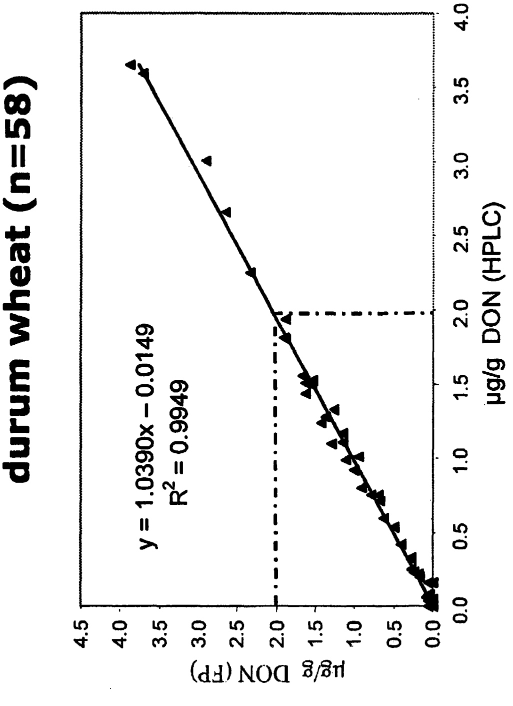
The optimized FP method for the determination of DON in wheat and derived products showed better sensitivity and accuracy with respect to the previously reported FP immunoassay (*34*) and better accuracy and precision with respect to the LC/immunoaffinity clean-up method (*37*). The assay is easy to perform, and may be useful for DON screening at levels that occur naturally in wheat and wheat based products.

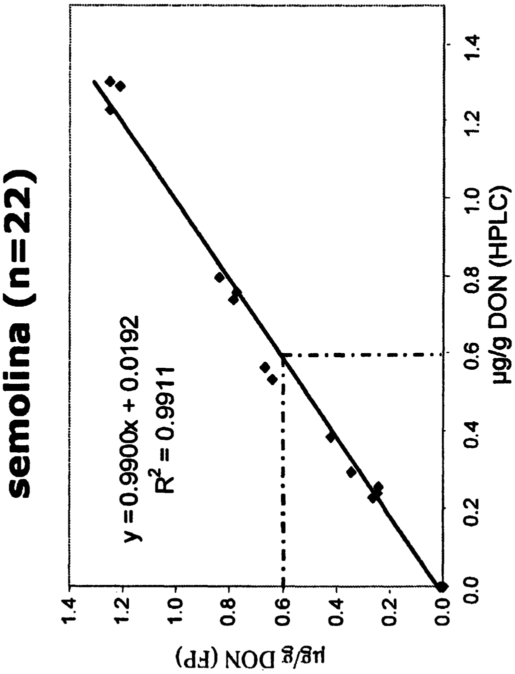
Summary and Future Research

Due to the increased need for detecting trichothecenes (mainly deoxynivalenol, T-2 toxin, and HT-2 toxin) in a variety of food products at low levels in order to carry out more reliable risk assessment studies for these toxins, rapid, sensitive, and accurate analytical methods for their determination are greatly required.

Recent researches carried out at ISPA-CNR (Bari, Italy) and at the Mycotoxin Research Unit of the USDA-ARS-NCAUR (Peoria, IL, USA) identified new sensitive fluorescent labeling reagents (commercially available) for T-2 and HT-2 toxins allowing their simultaneous determination by LC-FD. A new method has been then developed for the simultaneous determination of T-2 and HT-2 toxins in wheat, corn, and barley by LC-FD and 1-anthroylnitrile (1-AN), as labeling reagent, after immunoaffinity column clean-up with good accuracy, sensitivity, and precision. Furthermore, a Fluorescence Polarization (FP) immunoassay for rapid quantification of deoxynivalenol (DON) in common wheat, durum wheat, semolina, and pasta has been developed/optimized. The assay is easy to perform, and allows rapid and quantitative determination of DON at levels that can naturally occur in wheat and wheat based products. FP immunoassay showed better accuracy and precision with respect to a widely used LC/immunoaffinity clean-up method and can be used as alternative and less expensive method for DON analysis.

Future research efforts will be directed towards the development/optimization of LC-FD methods for the determination of T-2 and HT-2 toxins in a variety of processed cereal products (i.e., breakfast cereals, bread, biscuits, pasta, snack products, and baby foods) and the optimization of FP immunoassay for the determination of DON in additional matrices (i.e., maize, bran, processed cereal products, and baby foods).


durum wheat (n=58)
y = 1.0390x – 0.0149
R^2 = 0.9949
µg/g DON (FP)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0
4.5
µg/g DON (HPLC)
0.0
0.5
1.0
1.5
2.0
2.5
3.0
3.5
4.0


semolina (n=22)
y = 0.9900x + 0.0192
R^2 = 0.9911
µg/g DON (FP)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
µg/g DON (HPLC)
0.0
0.2
0.4
0.6
0.8
1.0
1.2
1.4

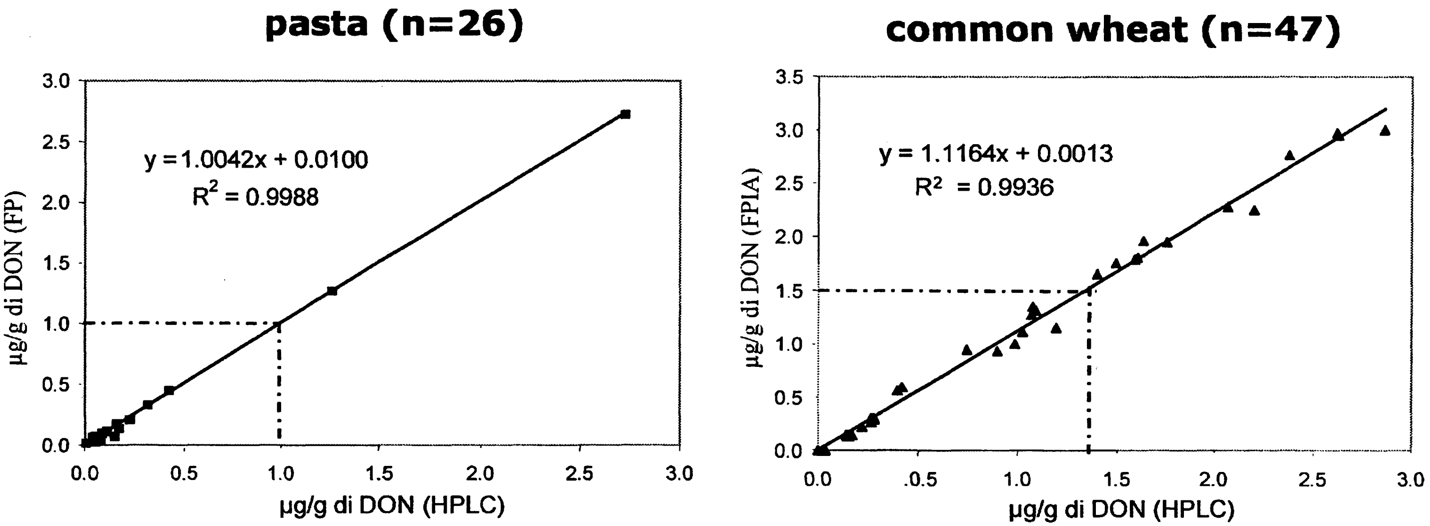


Figure 6. Comparison of DON contents in naturally contaminated samples of durum wheat, semolina, pasta, and common wheat analyzed by FP immunoassay and LC (data corrected for recovery).

Acknowledgment

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Disclaimer

Mention of trade names or commercial products in this article is solely for the purpose of providing specific information and does not imply recommenddation or endorsement by the U.S. Department of Agriculture.

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Chapter 11

Analysis of Trichothecenes Using Liquid Chromatography–Mass Spectrometry

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Trichothecenes are toxic secondary metabolites of various filamentous fungi and occur ubiquitously in agricultural produce and processed food and beverages. Trichothecenes are acutely cytotoxic and strongly immunosuppressive; toxicosis can be characterized by gastrointestinal disturbances, such as vomiting, diarrhea and inflammation, dermal irritation, feed refusal, abortion, anemia and leukopenia. The chronic effects of long-term low level exposure to these toxins are unknown, so monitoring of trichothecenes in food and animal feed at low concentrations has become particularly important. Many approaches have been adopted for the screening and detection of trichothecenes; however, approaches employing Mass Spectrometry (MS) and more recently Liquid Chromatography-Mass Spectrometry (LC-MS) have demonstrated the ability to provide both confirmatory and quantitative data. This chapter reviews the detection of trichothecenes using LC-MS.

In Food Contaminants; Siantar, D., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2008.

Trichothecenes are a large family of closely related sesquiterpenoid mycotoxins which are mainly produced by various species of *Fusarium, Trichoderma, Myrothecium, Stachybotrys, Cylindrocarpon,* and *Trichothecium*, the latter from which the trichothecenes were named. Trichothecenes constitute the largest group of mycotoxins, approximately 200 analogues, and are polycyclic sesquiterpenoids possessing a 12,13-epoxy-trichothec-9-ene ring system and various hydroxyl and acetoxy groups (*1*). The trichothecenes are classified into four groups (A-D) according to their functionality. Type-A trichothecenes include the highly toxic HT-2 toxin (HT-2), T-2 toxin (T-2) and diacetoxyscirpenol (DAS) and have a functional group at the C_8 position which is different from a keto-group. The type-B trichothecenes, such as deoxynivalenol (DON, also known as 'vomitoxin'), nivalenol (NIV), fusarenone-X (FUS-X), 3-acetyldeoxynivalneol (3AcDON) and 15-acetyldeoxynivalenol (15AcDON), differ from type-A by the presence of an α,β-unsaturated carbonyl group at the C_8 position (Figure 1). Trichothecenes containing two epoxide groups are classified as Type-C trichothecenes and all macrocyclic trichothecenes are classified as type-D trichothecenes.

Although the number of trichothecenes is very large, only a small number of these occur as natural contaminants of cereal products. Surveys have revealed that *Fusarium* fungi are ubiquitous, occurring on a broad variety of hosts. Cereals, especially maize and wheat are susceptible to infection in the field and during storage by these fungi. Field contamination of cereals depends heavily on environmental conditions such as moisture content of the substrate, temperature, soil type, and genetic susceptibility of cereal cultivars to fungal infection (*2*). Although *Fusarium* can be found worldwide they are particularly prevalent in regions with moderate climates. These plant pathogens grow parasitically on the cereal kernels and contaminate the plant tissue. Epidemiological studies have found that type-A and type-B trichothecenes are the most common trichothecenes that contaminate cereals. Macrocyclic trichothecenes rarely occur in cereal commodities. Many surveys have reported that DON, NIV, 3AcDON, 15AcDON are the most prevalent mycotoxins; HT-2 and T-2 are less frequently found and tend to be present in lower concentrations than DON. A study of 500 samples from 19 countries worldwide reported that 40-50% of samples were contaminated with DON, NIV, or zearalenone. The average concentrations for DON and NIV were 292 ng/g and 267 ng/g, respectively (*3*).

The diverse chemistry of the trichothecenes leads to a wide range of toxic effects in animals and humans. All trichothecenes possess an epoxide moiety which has been shown to play a role in their toxicity (*4, 5*). Type-A and type-B trichothecenes exhibit acute toxic effects such as vomiting, feed refusal, and weight loss. Type-A trichothecenes such as T-2, HT-2, and DAS can induce lesions on the mucosa of the mouth and esophageal region in swine and poultry. (*6, 7*). Moreover, type B trichothecenes have shown more chronic toxic effects, including inflammatory responses, extensive hemorrhaging as well as hematological toxicities. Type-B trichothecenes also inhibit protein and DNA

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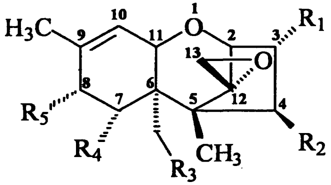
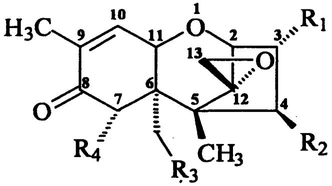


Type A Trichothecenes

	MW	R_1	R_2	R_3	R_4	R_5
T-2 Toxin	466	OH	OAc	OAc	H	$OCOCH_2CH(CH_3)_2$
HT-2 Toxin	424	OH	OH	OAc	H	$OCOCH_2CH(CH_3)_2$
Acetyl T-2 Toxin	508	OAc	OAc	OAc	H	$OCOCH_2CH(CH_3)_2$
Monoacetoxyscirpenol	324	OH	OH	OAc	H	H
Diacetoxyscirpenol	366	OH	OAc	OAc	H	H
Triacetoxyscirpenol	408	OAc	OAc	OAc	H	H
Neosolaniol	312	OH	OAc	OAc	H	OH



Type B Trichothecenes

	MW	R1	R2	R3	R4
Nivalenol	312	OH	OH	OH	OH
Deoxynivalenol	296	OH	H	OH	OH
Fusarenone-X	354	OH	OAc	OH	OH
3-Acetyldeoxynivalenol	338	OAc	H	OH	OH
15-Acetyldeoxynivalenol	338	OH	H	OAc	OH

Figure 1. Structures of trichothecene mycotoxins

synthesis, mitochondrial function *in vitro* and *in vivo*, and show immunosuppressant effects, even at low concentrations (*8-10*). Due to the polar nature of the trichothecenes, they are rapidly absorbed and metabolized after ingestion (*11*); they are also mainly excreted in the urine and to a lesser degree in bile (*12*). Swine are among the most sensitive animals to trichothecenes while ruminants are able to detoxify trichothecenes. DOM-1 (a DON metabolite) is formed from DON by gut and rumen bacteria through reduction of the epoxide group of DON. This de-epoxidation leads to a significant reduction in toxicity. Where two or more mycotoxins that share the same toxicological effects are found together additive effects can occur. However, synergistic effects have also been reported when multiple trichothecene analogs co-occur (*6, 13*).

Although the high toxicity of the trichothecenes has been well documented, few countries have established legal regulations or recommendations for these compounds. The European Scientific Committee on Food (SCF 2002) set provisional Tolerable Daily Intakes (TDI) for DON (1 μg/kg bodyweight/day) and combined T-2 and HT-2. (0.06 μg/kg bodyweight/day) (*14*). European screening studies have shown that the TDI for combined T-2 and HT-2 intake is often exceeded. These higher intakes were particularly significant for infants and children.

Since *Fusarium* toxins are so widespread and have such a toxic effect, there is a need for rapid and reliable means of identification and quantitation in cereals and foodstuffs in order to control both acute and chronic exposure, and to meet current and forthcoming legislation. Identification and quantitative assessment of trichothecenes generally requires detailed sampling protocols, elaborate sample preparation and sensitive detection techniques. The natural coexistence of several mycotoxins in cereals and related products also requires the simultaneous analysis of these substances.

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LC-MS Analysis of Trichothecenes

The analysis of trichothecenes generally requires a separation technique such as thin layer chromatography (TLC), gas chromatography (GC) or liquid chromatography (LC), coupled with one of a variety of detection techniques. Comprehensive reviews on the analytical methods used for trichothecene analysis have been published previously (*15, 16*). LC with ultra-violet (UV) detection has been used for the determination of some trichothecenes. DON and NIV have been detected down to levels of 50 μg/g and 15 μg/g, respectively, without derivatization (*17*). However, the disadvantage of LC-UV analysis is that most of the trichothecenes do not contain a natural chromophore, therefore a derivatization step must be included to expand the range of toxins that can be determined. Mass spectrometric analysis allows a much broader range of trichothecenes to be determined and, for this reason, most applications have involved GC-MS (*16*). Although there have been some reports of using GC-MS for trichothecene determination without derivatization, significant adsorption of the polar trichothecenes to the column has been reported. Derivatization has been necessary for GC-MS analysis, both to prevent adsorption of the compounds on the analytical column and to make the thermally labile trichothecenes more volatile, thus allowing sensitive trace analysis. The need for derivatization is the greatest drawback to GC-MS analysis.

Because chromatographic resolution of trichothecenes can be achieved without the use of derivatization, LC has emerged as the method of choice for the analysis of multiple trichothecene analogs. Coupling of LC and mass spectrometry (MS) instruments provides a powerful tool in mycotoxin analysis. LC-MS/MS has been applied to mycotoxin analysis despite greater costs. The main advantages of this technique include its general applicability to a broad range of compounds, high sensitivity and outstanding selectivity. LC-MS instruments, particularly using Atmospheric Pressure Chemical Ionization (APCI) and Electrospray Ionization (ESI) interfaces have recently been employed for the determination and identification of trichothecenes at trace levels. Furthermore, Multiple Reaction Monitoring (MRM) with LC-MS/MS instruments gives enhanced performance, providing additional selectivity and increased sensitivity (based on signal-to-noise) over a wide linear range.

The main obstacle facing LC-MS analysis is the effect of matrix components on the analyte signals. The initial idea that APCI with tandem mass spectrometry was a simple solution for complex analytical problems has been revised. More and more experimental evidence shows that, especially for multicomponent analysis in complex samples, the matrix can affect (usually weakening) the analyte signal to a large extent. However, recent developments in ionization sources have dramatically improved the ability of LC-MS to analyze thermally labile, intractable polar and ionic analytes without chemical derivatization. Table I outlines the MS analyzers and the LC-MS interfaces that have been applied to the analysis of trichothecenes.

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Extraction of Trichothecenes

In the development of analytical protocols for the analysis of trace contaminants, there is an inevitable conflict between the need to extract the target analytes efficiently and to limit the co-extraction of non-target and potentially interfering components. As a consequence, a combination of an efficient solvent extraction procedure and a sample clean-up step is usually employed for trichothecene determination.

Various approaches have been adopted for extraction of trichothecenes from a wide variety of matrices, ranging from foodstuffs and grains to building materials. Initially, Scott *et al.* (*18*) extracted samples with methanol-water (1:1, v/v) but this was later modified to methanol-water (7:3, v/v) (*19, 20*) and methanol-water (9:1, v/v) (*21, 22*). However, because of the emergence of

Table I. Mass Analyzers and Ionization Modes Used for the LC-MS Analysis of Trichothecenes

Mass Analyser	*Ionization Mode*	*Trichothecenes Assayed*	*Sample Matrix*	*Ref.*
QIT	APCI (+)	NIV, DON, Fus-X, 3-AcDON, 15-AcDON, NEO, DAS, HT-2, T-2	Wheat	(*31*)
QqQ	APCI (switching)	NIV, DON, Fus-X, 3-AcDON, 15-AcDON, NEO, HT-2, T-2	Maize	(*33*)
QqQ	ESI (switching)	NIV, DON, Fus-X, 3-AcDON, 15-AcDON, DAS, HT-2, T-2, verrucarrol (VOL)	Flour, breads, grains, corn, soy products, breakfast snacks	(*29*)
QqQ	ESI (switching)	NIV, DON, Fus-X, 3-AcDON, 15-AcDON, NEO, MAS, DAS, HT-2, T-2	Corn meal	(*36*)
QqQ	ESI (-)	NIV, DON, Fus-X, 3-AcDON, 15-AcDON	Maize	(*51*)
Single Quad.	ESI (+)	NIV, DON, Fus-X, 15-AcDON, NEO, DAS, HT-2, T-2	Wheat, maize	(*47*)
QqQ	ESI (+)	DON, DAS, T-2	Oats, cornmeal, feedstuffs	(*28*)
QqQ	APCI (+)	DAS, HT-2, T-2	Poultry feed	(*52*)
QqQ	APCI (-)	NIV, DON, Fus-X, 3-AcDON	Maize	(*35*)
TOF	ESI (+)	474 Toxins and metabolites	Fungal culture extracts	(*49*)
QIT	ESI (-)	DON	Grains, food products	(*34*)
QIT	ESI (-)	DON, NIV	Corn, wheat	(*46*)
Single Quad.	APCI (-)	NIV, DON, Fus-X, 15-AcDON, 3-AcDON	Pig urine, maize.	(*26*)
Single Quad.	APCI (+)	NEO, DAS, MAS, HT-2, T-2, Acetyl T-2	Oats, maize, barley, wheat	(*32*)
Single Quad.	APCI (-)	DON, NIV	Wheat	(*43*)
QIT	APCI (switching)	DON	Maize	(*45*)

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Table I. *Continued.*

Mass Analyser	*Ionization Mode*	*Trichothecenes Assayed*	*Sample Matrix*	*Ref.*
QqQ	ESI (switching)	DON, 15-AcDON, 3-AcDON, NIV, Fus-X, T-2.	Rice.	(*30*)
QqQ	ESI (-)	DON.	Egg.	(*53*)
TOF	APCI (+)	NIV, DON Fus-X, 3-AcDON, 15-AcDON, DAS, HT-2, T-2.	Wheat, biscuits, corn, cornflakes.	(*50*)
QIT	ESI (+)	T-2 Tetraol, VOL, DAS, T-2 Triol, HT-2, T-2.	dust, gypsum board, carpet, wood.	(*27*)
Single Quad.	TSP-MS	T-2 Tetraol, DON, DAS, HT-2, T-2.	Urine, plasma.	(*37*)
Single Quad.	TSP-MS	DON, MAS, T-2 Triol, T-2 Tetraol, NEO, HT-2, T-2.	Feces, urine.	(*38*)

customized solid phase extraction (SPE) columns, for example MycoSep, as the popular means of sample clean-up, the accompanying extraction with acetonitrile-water (84:16, v/v) has been widely adopted by many laboratories for trichothecene analysis. It has been demonstrated that using acetonitrile-water (84:16, v/v) results in the lower co-extraction of interferants when compared with methanol-water (1:1, v/v) (*23*).

Tanaka *et al.* (*24*) showed that the highest recovery of NIV and DON were obtained using acetonitrile-water (3:1, v/v); lower recoveries were achieved with acetonitrile-water (3:2, v/v) and methanol-water (3:1, v/v). Extractions using acetonitrile-water (9:1, v/v) showed a 20% reduction in recoveries.

Other solvent systems applied to the extraction of trichothecenes have included ethyl acetate-acetonitrile-water (20:5:1, v/v/v). Möller *et al.* (*25*) showed that extraction with this combination gave cleaner extracts than those obtained using acetonitrile or methanol and water. The choice of extraction solvent is also influenced by the method of sample clean-up to be used subsequent to the extraction step. The use of acetonitrile-water (84:16, v/v) for extraction allows direct transfer of the crude extract onto MycoSep columns. Razzazi-Fazeli *et al.* (*26*) extracted trichothecenes from pig urine using 100 % acetonitrile before clean-up on a MycoSep 227 column. Tuomi *et al.* (*27*) used 95% methanol to extract trichothecenes from building materials before clean-up on Bond Elute and Isolute SPE cartridges.

The extraction efficiencies are influenced by the blender or homogenizer speed and also by the shape and size of the extraction flasks used. It has been demonstrated that the extraction times required for naturally contaminated samples

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can be much greater than those required to extract spiked samples. Trenholm *et al.* (*23*) showed that samples naturally contaminated with DON can require up to 16 min of blending for extraction whereas spiked samples could be extracted in as little as 3 min when using acetonitrile-water (84:16, v/v).

Supercritical fluid extraction (SFE) has also been investigated as a means of extracting trichothecenes from grains and feed. Huopalahti *et al.* (*28*) used carbon dioxide, with 5% methanol, at 550 atm pressure and 60 °C for LC-MS analysis and reported recoveries better than 85 %.

Sample Clean-up

Since LC-MS techniques are influenced by the so-called matrix effects, a sample clean-up step is usually required. Matrix effects influence the yield of analyte ions, usually suppression of ionization efficiency due to the presence of co-extracted compounds. Experimental evidence shows that, especially for multi-component analysis in complex samples, the matrix effect can weaken the ionic signal to a large extent. Biselli *et al.* (*29*) demonstrated that matrix components extracted from soy beans can suppress the signal given by DON by up to 40% (Figure 2).

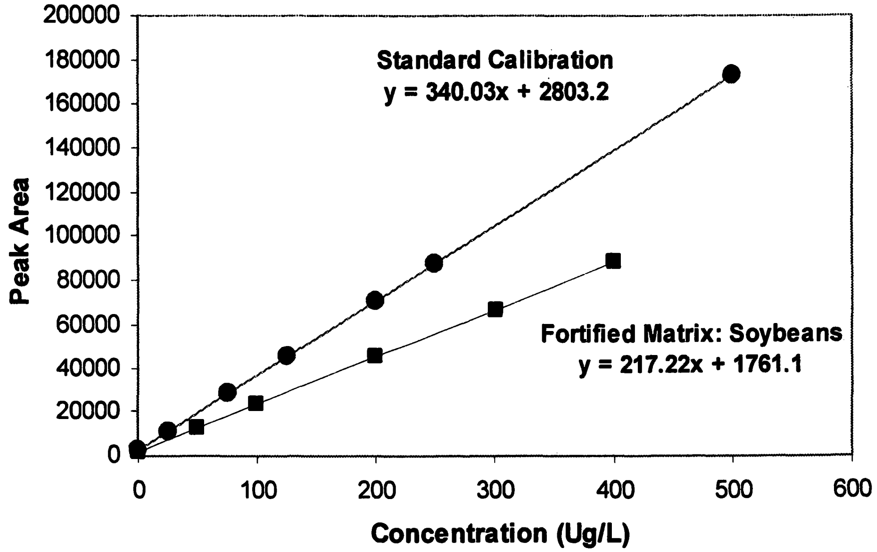

Figure 2. Calibration graphs demonstrating the extent of ion suppression produced in matrix-matched standards of DON using LC-ESI-MS/MS [Adapted from Biselli et al. *(29) with permission. Copyright (2005) Taylor & Francis Ltd., http://tandf.co.uk/journals].*

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Sagawa *et al.* (*30*) demonstrated that as sample matrix concentration increased, the peak area of the trichothecenes was reduced and the peak area of T-2 became more variable. The co-extractives contained in many of the matrices, typically lipids and pigments, demand an efficient clean-up procedure. Efficient sample clean-up can reduce the ion suppression phenomena leading to increased sensitivity, preserves a clean LC-MS interface, and enables a higher sample throughput in LC-MS.

The most widely adopted method of sample clean-up for LC-MS analysis of trichothecenes is the MycoSep column. These multifunctional clean-up columns consist of a packing material which contains various sorbents such as charcoal, Celite, ion-exchange resins, and more. Berger *et al.* (*31*) reported that the MycoSep clean-up procedure they employed almost completely removed the sample matrix interferences allowing free MS detection; internal standards were used to show that no matrix effects were observed when sample extracts of grains were subjected to clean-up using MycoSep columns (*32*). It has been reported that, with the exception of (NIV), the MycoSep #226 column clean-up performed excellently (recoveries of 82-98 %) for both Type-A and Type-B trichothecenes. It was also reported that MycoSep #226 allowed compounds of a wider polarity range to pass through the column but this would also result in the elution of more matrix compounds (*33*). Generally, polar trichothecenes such as NIV and DON, show poorer recovery on MycoSep columns while the less polar compounds such as T-2 perform better. However, by using an additional rinse step the recovery rates for all trichothecenes were improved (>80 %) without increasing the background matrix (*31*). Razzazi-Fazeli *et al.* (*26*) showed that MycoSep columns also show the ability to provide good recovery (*ca.* 96%) of trichothecene metabolites such as DOM-1, the DON metabolite.

Biselli *et al.* (*29*) reported that the use of MycoSep clean-up columns was a necessary step and that any reduction in the sample preparation resulted in unacceptably high matrix effects. However, some laboratories using LC-MS have experimented with direct analysis of sample extracts. When the use of a MycoSep clean-up column was compared with direct analysis of sample extracts for DON, it was shown that direct analysis gave a Limit of Detection (LOD) < 2 ppm but ten-fold lower LODs could be achieved when the MycoSep clean-up was applied (*34*).

Other forms of sample clean-up have also been applied to the LC-MS analysis of trichothecenes. Lagana *et al.* (*35*) experimented with a graphitized carbon black (GCB) Carbograph-4 material. This material has reverse phase sorbent, polar interaction, and anion exchanger properties; moreover it also possesses a particular affinity for aromatic compounds with respect to aliphatic ones. By exploiting this property, separation of compounds can be achieved by differential elution, by passing suitable eluent phases sequentially through the cartridge. Cavaliere *et al.* (*36*) carried out a comparison of Carbograph-4 cartridges and Oasis HLB cartridges. The Oasis HLB cartridges are filled with

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hydrophilic-lipophilic balanced sorbents. Recoveries for both materials were comparable (> 82 %) for most of the analytes studied; however, NIV, Fus-X, NEO, and the AcDON's were insufficiently recovered from the Oasis HLB cartridges (27-54 %). The eluents from the Oasis HLB cartridges also exhibited stronger matrix effects than those collected from the Carbograph-4 columns.

Tuomi *et al.* (*27*) investigated the presence of trichothecenes in building materials after liquid/liquid portioning of the extracts with hexane and SPE. Bond Elut C_{18}, Isolute C_{18}, Isolute MF C_{18} (non-end capped), Isolute C_8 (end capped), Isolute C_8 (non-end capped), and Isolute ENV^+ stationary phases were investigated. The most polar toxins did not adhere properly to the non-end capped materials and showed even poorer adhesion to the less polar C_{18} materials. The non-end capped C_8 solid phase sorbent was shown to work best for the suite of toxins analyzed.

Voyksner *et al.* (*37*) performed direct sample clean-up of urine and plasma using Clin-Elut Columns in conjunction with C18 Sep Pak and Silica Gel columns. This method demonstrated LOD's of 0.5-20 ng using a ThermoSpray LC-MS interface.

Ionization Techniques

Early Ionization Sources

ESI and APCI are modern means of interfacing LC and MS instruments. In the early studies of the use of LC-MS for analysis of trichothecenes, Fast Atom Bombardment (FAB), Thermospray (TSP), and moving belt interfaces were used. However the Thermospray interfaces were the most widely adopted ionization source.

Voyksner *et al.* (*37*) showed that the addition of an aqueous solution containing ammonium acetate increased the TSP sensitivity. The TSP ionization appears to become more efficient with increasing water content. However, since LC conditions cannot be easily varied to accommodate a larger percentage of water, the addition of the aqueous buffer solution was made post-column. This enhanced the signal level for most toxins by a factor of 2-3 while maintaining chromatographic integrity. Voyksner *et al.* (*38*) also experimented with the TSP source in both ion evaporation and filament on Chemical Ionization (CI) mode. The trichothecene spectra acquired in CI mode were nearly identical to those obtained under Thermospray ion evaporation conditions for both negative and positive ion detection. This showed that the gas phase reactions of the buffered LC solvent resulted in the same ions in both modes. However the CI spectra were shown to be 15-40 times more sensitive than Thermospray spectra in positive ion mode, and 2-3 orders of magnitude more sensitive in negative ion mode. The operation of CI in positive ion mode was also shown to be 2-5 times

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more sensitive than negative mode. The superior sensitivity of positive ion CI mode and the increased abundance of fragment ion formation, resulting in increased specificity, aided the identification of unknowns. Thus, positive CI was shown to be the best mode of operation for the analysis of trichothecenes. This approach was adopted to identify and verify the presence of trichothecenes and their metabolites in several different samples. Using this method, Voyksner *et al.* (*38*) reached LOD values of 50-1000 pg on-column. Analysis of urine from cows fed grain contaminated with DON was accomplished by LC-MS to detect the free and glucuronide conjugates of DON and DOM-1 (a DON metabolite). Unlike GC-MS, LC-MS has the potential to detect both free and conjugated toxins in a single analysis without derivatization. Voyksner *et al.* (*38*) also showed that plasma or urine matrix does not increase the chemical noise content of the chromatogram since TSP analysis was not hindered by the presence biological compounds which are insensitive to TSP ionization. Supercritical Fluid Chromatography (SFC)-MS using a moving belt interface has been used for trichothecene but with poor sensitivity (*39*).

Krishnamurthy *et al.* (*40*) used ammonium acetate buffer as a modifier in the LC mobile phase to generate $[M+NH_4]^+$ ions which were then subjected to Collision Induced Dissociation (CID) experiments. These experiments showed that when the ammonium ions were subjected to CID, protonated molecular ions formed initially before further fragmentation. The CID spectra of the corresponding protonated molecules were also obtained with lesser sensitivity under the same conditions to yield the same product ions.

A comparative study of TSP and FAB ionization with regard to trichothecenes has been performed. It was noted that FAB ionization gave rise to much more fragmentation when compared with TSP. This lack of fragmentation was due to the low exothermicity of the ionization process in the ammonium acetate buffered TSP, owing to the high proton affinity of ammonia. The more extensive fragmentation in FAB indicated a more energetic ionization process. The formation of abundant fragment ions in FAB allows reliable detection of the trichothecenes by selected ion monitoring (SIM). However, the energetic ionization process may lead to decreased selectivity for detection of the trichothecenes in complex matrices where the number of interfering ions from background compounds is increased (*41*). Kostiainen *et al.* (*42*) also investigated the fragment ions produced by CID experiments with FAB ionization. This work showed fragment ions are formed by the losses of functional groups as neutral species (e.g., water, acetic acid, formaldehyde, and isovaleric acid) in various combinations. It was noted that the most abundant fragment ions were formed by the loss of isovaleric acid and/or by successive losses of units of acetic acid. Triacetoxyscirpenol (TAS), which does not contain any hydroxyl group, demonstrated the loss of water. It was suggested that this water loss originated from the acetoxy group. This suggests the possibility that the ions $[M+H-H_2O]^+$ are partly formed by the loss of water from the acetoxy or isovaleroyloxy groups in the other trichothecenes.

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Atmospheric Pressure Chemical Ionization (APCI)

Currently, APCI and ESI are the most widely used LC-MS interfaces for trichothecene analysis. Using APCI, Berger *et al.* (*31*) was able to achieve LOD values of 20-120 pg on-column for trichothecenes. They also reported that the APCI source caused limited fragmentation which allows low detection limits to be reached. The predominant trichothecene ions in APCI positive mode were $[M+H]^+$ and $[M+NH_4]^+$ ions; occasionally $[M+CH_3OH+H]^+$ adducts were observed. Traces of ammonium generally present in the background generate an $[M+NH_4]^+$ adduct with an abundance of 10-50 % relative to the base ion $[M+H]^+$ for trichothecenes with an acetyl group at C_{15}. A post-column addition of ammonium acetate produced mass spectra containing almost exclusively the $[M+NH_4]^+$ ion. On the other hand, trichothecenes without an acetyl group at the C_{15} position were not detectable in APCI positive mode after increasing the ammonium level. In APCI negative mode, the addition of ammonium acetate resulted in spectra of type-B trichothecenes showing exclusively the [M-acetate]$^-$ ion. As a result, the signal-to-noise (S/N) ratios increased by a factor of 3, 5, 5, 10, and 12, respectively for 3-AcDON, 15-AcDON, Fus-X, NIV, and DON. Type-A trichothecenes did not form anions in the presence or absence of ammonium acetate. Therefore, it has been postulated that the addition of acetate ion occurred at the α,β-unsaturated ketone of the type-B trichothecenes (*31*).

Razzazi-Fazeli *et al.* (*43*) operated the APCI source in negative ion mode and in contrast to the results of Berger *et al.* (*31*), a decrease in the signal-to-noise (S/N) ratio was observed when using modifiers in the LC mobile phase. In the case of ammonium acetate, the signal was strongly suppressed and the detection limit was reduced. The addition of ammonia, as a deprotonization agent, had no significant effect on the signal intensity while the addition of acetic acid to the mobile phase showed a negative effect on the S/N ratio. When buffers were added to the LC mobile phase, adduct ions of either acetate or ammonia were observed in all experiments. The effect of vaporization temperature on the ion abundance of trichothecenes has also been investigated. Under TSP conditions the ionization is carried out at a temperature of about 250 °C and no strong thermal degradation was reported in these studies. Using the APCI interface, much higher temperatures (>300 °C) have to be applied for a better ionization. The increase of the vaporizing temperature leads to a reduction of the aerosol droplet sizes and yields an improvement in the evaporation of the solvent and therefore better ionization efficiency. Varying the vaporizer temperature between 300 and 500 °C, the optimal temperature for a maximum response was found for NIV at about 400 °C and for DON at 350 °C. The intensities of the deprotonated molecules $[M–H]^-$ and the adduct ions $[M–H–2H_2O]^-$ decrease as the vaporizer temperature is increased. Generally higher temperatures lead to intensive fragmentation as a result of thermal degradation (*43*).

The optimum vaporizer temperature is naturally influenced by the flow-rate of the LC eluent. For both substances tested (NIV and DON), the optimum LC

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flow-rate was found to be between 0.9 and 1 mL/min. The optimal flow-rate for signal intensity depends on the interface design and the geometry of the interface as well the chemical properties of each individual compound (*43*).

Razzazi-Fazeli *et al.* (*32*) showed that the response of type-A trichothecenes was distinctly more sensitive in negative ion mode. It was also shown that APCI ionization gave spectra with more intense fragmentation than those acquired using TSP. Intensive ammonium adducts were formed for those trichothecenes with ester groups at the C_{15} position, and additionally it was also shown that trichothecenes with ester groups at the C_4 position exhibited a strong tendency to form ammonium adducts. However HT-2 and monoacetoxyscirpenol (MAS), which only have one ester group at C_{15} and C_4, respectively, produced $[M+H]^+$ ions and not ammonium adducts. Because these compounds contained ester groups at the C_{15} and C_4 positions, respectively, they were expected to show a strong tendency to form ammonium adducts. This showed that the formation of $[M+NH_4]^+$ adducts is due to the number of ester groups and does not depend on their position in the molecule (*32*). These results were in accordance with Rajakylä *et al.* (*44*). As a result, the ionization mechanisms in APCI seem to be very similar to the ionization mechanisms for TSP ionization. Typically, it is difficult to predict the behavior of molecules in an LC-MS interface. This was also demonstrated by observing the behavior of the DON derivatives, 3-AcDON and 15-AcDON (*26*). These compounds differ only in the position of the acetate substituent but the response of these compounds in LC-APCI-MS differed significantly: 50 ng/g for 3-AcDON and 150 ng/g for 15-AcDON. Similar behavior was reported by Royer *et al.* (*45*) when operating the APCI source in positive ion mode. The positive APCI mass spectrum of DON showed a single intense ion at *m/z* 296 corresponding to the molecular mass of the compound. This was a surprising result, as the protonated molecule (*m/z* 297) was expected as reported by Berger *et al.* (*31*). Different investigations of the mobile phase percentage, with and without ammonium acetate, led to the observation of a small amount of the ammonium adduct ion (*m/z* 314). Thus, the presence of *m/z* 296 was postulated as due to an unstable ammonium adduct ion that subsequent lost a water molecule leading to *m/z* 296. This behavior was compared with that of VOL, which has a very close chemistry to that of DON. Under the same conditions VOL was detected as a protonated molecular ion at *m/z* 267 with a much lower response than that of DON. It was therefore suggested that the absence of both the keto C_8 and hydroxyl C_7 groups might stabilize the protonated molecule (*43*). These observations underline the substance specificity of an LC–MS interface.

Berthiller *et al.* (*33*) showed that, depending on the structure of the trichothecene, $[M-H]^-$, $[M+HCOO]^-$ and $[M+CH_3COO]^-$ ions in negative mode and $[M+H]^+$, $[M+NH_4]^+$, $[M+Na]^+$ as well as $[M+K]^+$ ions in positive mode are formed. Using buffers, the adduct ion formation could be shifted towards $[M+NH_4]^+$ ions in positive mode for the type-A trichothecenes and to either $[M+HCOO]^-$ or $[M+CH_3COO]^-$ ions in negative mode for the type-B

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trichothecenes. Since the use of a buffer can lead to the formation of single trichothecene adduct ions, the intensities of these ions were much higher compared to experiments without buffers. Using ammonium acetate, the peak areas of both $[M+NH_4]^+$ ions for type-A trichothecenes in positive ionization mode and $[M+CH_3COO]^-$ ions for type-B trichothecenes in negative ionization mode were approximately equivalent to the sum of the different analyte adduct ions when using no buffer at all. In general, type-A trichothecenes produced stronger MS signals as positive ions while type-B trichothecenes showed higher signal intensities in the negative ionization mode. For this reason Berthiller *et al.* (*33*) applied polarity switching to the APCI source. This allowed the maximum intensities for each trichothecene analyzed to be reached by switching between positive ionization mode and negative ionization mode where appropriate (Figure 3).

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Electrospray Ionization (ESI)

Until recently, few methods were reported using ESI for the analysis of trichothecenes. This is probably due to the increased matrix effects encountered when using ESI. It is also difficult to achieve simultaneous detection of the type-A and type-B trichothecenes. As mentioned earlier, the less polar type-A trichothecenes respond better to positive ionization (*32*), whereas type-B trichothecenes respond better to negative ionization (*43*). The difference is probably due to the ability of the type-B trichothecenes to efficiently delocalize a negative charge due to enolate formation.

Plattner *et al.* (*46*) compared the performance of DON and NIV in wheat extracts with both ESI and APCI sources. In mixtures of water-methanol, negative ESI yielded less abundant signals for NIV than were seen using APCI conditions. The negative ESI spectrum of DON was similar to that reported for negative APCI, a result supported by Tuomi *et al.* (*27*) who showed that methanol was the preferred modifier even though for some chromatographic purposes acetonitrile would have been preferred. This was due to the fact that water content of more than 20%, would have impaired ionization using ESI. However, Plattner *et al.* (*46*) showed that when the APCI interface was used in positive ion mode, DON was protonated to yield a signal at *m/z* 297, together with fragment ions. In negative ionization mode, DON gave an ion at *m/z* 295 and it was observed that the addition of acetic acid to the solvent system resulted in the formation of intense acetate adduct ions at *m/z* 355 instead of the ion at *m/z* 295. Similarly, in ESI negative mode the adduct at *m/z* 355 $[M+CH_3COO]^-$ was detected. Addition of even trace amounts of acetic acid to the solvents resulted in a large increase in the signal for DON and NIV making the overall sensitivity of them approximately equal to that observed with APCI. While APCI and negative mode ESI give similar detection limits for DON, ESI was used for routine DON assays because it was found that the ESI source was much more

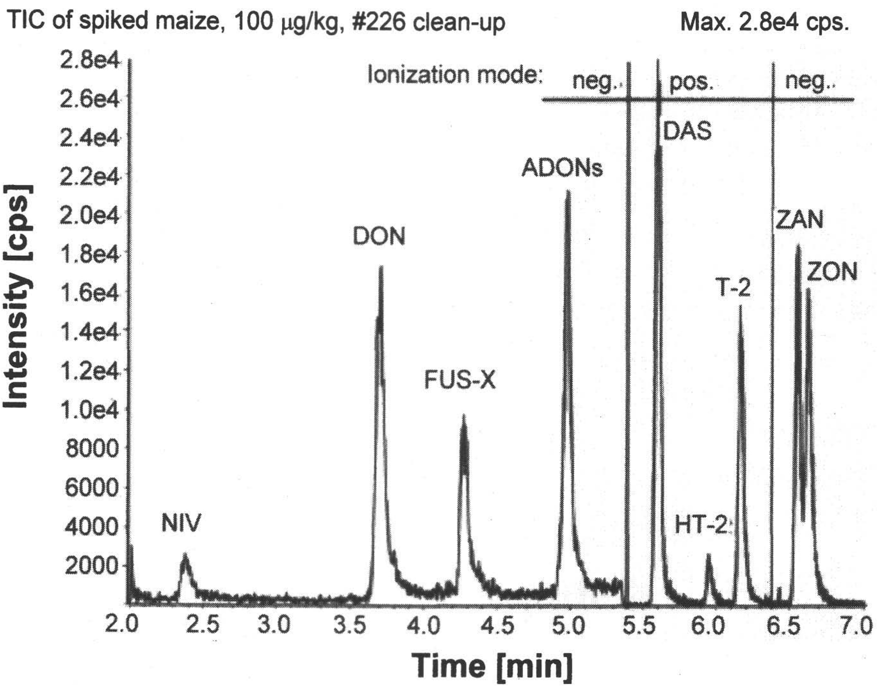

Figure 3. Maximum signal intensity was achieved by employing polarity switching during the chromatographic run [Reprinted from Berthiller et al. *(33) with permission. Copyright (2005) Elsevier].*

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rugged and forgiving when dirty samples were analyzed and the long term performance of the ESI interface was significantly better for injection of sample extracts. The ESI interface maintained sensitivity after many injections of dirty samples and required less cleaning and maintenance than APCI to maintain good performance. The APCI interface required rigorous cleaning every few days to maintain sensitivity while the ESI source gave satisfactory results with no significant loss of sensitivity for several weeks. ESI also has the advantage of lower background signals from solvents and sample matrices than are observed for APCI. It was found that the best full scan signal to noise (S/N) ratios for DON and NIV present in naturally contaminated extracts were obtained with ESI.

Biselli *et al.* (*29*) evaluated the suitability of the two different interfaces using a standard mix containing NIV, DON, VOL, zearalanone (ZAN) and zearalenone (ZON) at concentrations of 200 µg/L which was injected through each interface. Altogether, the application of ESI (TurboIonSpray) resulted in better response for all investigated compounds compared with Heated Nebulizer (APCI) technique. The comparison showed a 6-17 times higher response when the ESI interface was used and similar results were obtained by Lagana *et al.* (*35*). In contrast, Berthiller *et al.* (*33*) reported the best results using the APCI interface in combination with an ammonium acetate buffer.

Lagana *et al.* (*35*) showed that when using ESI, the $[M-H]^-$ ions of type-B trichothecenes were found to be more abundant than the corresponding $[M+H]^+$ ions. It was suggested that the presence of acid phenol groups favor deprotonation of the molecules in the gas phase. The addition of mobile phase modifiers was also examined and for almost all of the type-B trichothecenes studied, acidic additions led to significant enhancement of the $[M+H]^+$ ions, while in negative ion mode it strongly reduced the signal intensity. The addition of ammonium acetate, on the other hand, decreased the $[M+H]^+$ ion signal and in the negative ion mode it had only a small effect on $[M-H]^-$ signals.

Because of the tendency of type-A trichothecenes to form positive ions and type-B trichothecenes to form negative ions, different approaches have been taken to achieve simultaneous determination of both types of trichothecene. Tuomi *et al.* (*27*) showed that positive ion mode was clearly more effective than negative ionization mode for the trichothecenes. This was partially due to the strong tendency of these compounds to form sodiated adducts in ESI. Protonated molecules could not be detected at all, or were present in very low abundance, regardless of whether sodium acetate was added to the LC eluent or not. This was also observed by Dall'Asta *et al.* (*47*) who showed that type-A trichothecenes and some type-B trichothecenes had a tendency to form adducts with Na^+ and K^+ ions in positive mode. After the inclusion of sodium chloride as a mobile phase additive, the type-A trichothecenes spectra showed a gain in sensitivity and the disappearance of any molecular adducts other than the $[M+Na]^+$ adduct. The addition of sodium chloride was also very effective on the positive mode detection of the type-B trichothecenes. All sodium adducts were shown to be very stable, as expected, since sodiated adducts are less susceptible to fragmentation than their protonated analogs. For all toxins in the study, the LODs were found to be similar to those presented for detection by LC-APCI-MS.

As with APCI, the technique of polarity switching has also been adopted in ESI. Biselli *et al.* (*29*) used this technique to allow simultaneous determination of a wide range of different mycotoxins. As also shown by Razzazi-Fazeli *et al.* (*32*), the best sensitivity for type-A trichothecenes was achieved with positive ionization in ESI mode, while for type-B trichothecenes negative ionization was preferred.

ESI now appears in the literature to the same extent as APCI. This is because ESI has emerged as a real alternative to APCI since the former has the ability to maintain good sensitivity over a long working period, and the ability to resist matrix effects. For these reasons, ESI now appears to be more widely used in trichothecene determination than APCI, particularly with the advent of polarity switching.

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Atmospheric Pressure Photospray Ionization (APPI)

To-date no reports of the application of APPI to trichothecene analysis have been presented. However experiments conducted by the present authors showed that APPI does not produce ion abundances comparable with ESI. The strongest optimized molecule-related ions of each compound in each ion source were compared. The data is presented below (Figure 4).

These results showed that ESI was by far the most efficient ionization source, APCI produced ions for all the trichothecenes studied and APPI was able to match the performance of APCI for only some trichothecenes. However, APPI failed to produce useful ion abundances for five of the trichothecenes which suggests that APPI does not easily lend itself to the analysis of these compounds.

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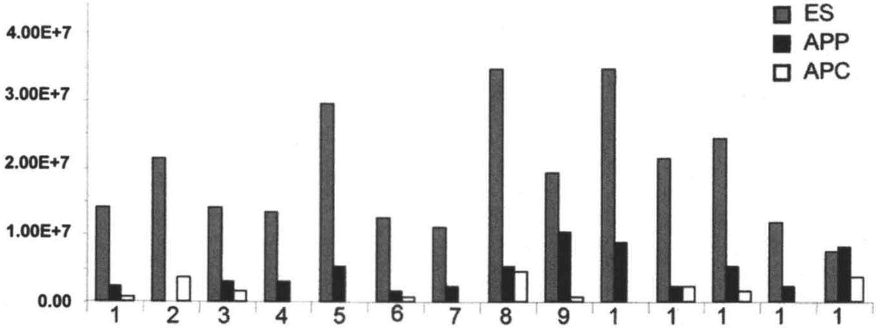

Figure 4. Comparison of ESI, APCI, and APPI ionization sources. The ion abundance of the strongest molecular ion in each ion source was tabulated for each toxin. 1. DON, 2. Fus-X, 3. Verrucarin A, 4. T-2 Tetraol Tetraacetate, 5. T-2, 6. HT-2, 7. T-2 Triol, 8. 4,15 Diacetylverrucarol, 9. DAS, 10. Neosolaniol, 11. Verrucarol, 12. Roridin A, 13. T-2 Tetraol, 14. 3-acetyldeoxynivalenol.

Mass Analyzers

All of the common mass spectrometry methodologies, Quadrupole Ion Trap (QIT), Single Quadrupole, Triple Quadrupole (QqQ) and Time-of-Flight (TOF) MS have been applied to trichothecene analysis. As in most analysis of food contaminants, the greatest obstacle facing LC-MS analysis of trichothecenes is the effect of co-extracted matrix components. For this reason, a universally acceptable Internal Standard (IS) is needed. Berger *et al.* (*31*) was among the first to suggest an IS for trichothecene analysis. VOL, a semi-synthetic trichothecene obtained by the hydrolysis of macrocyclic precursors, was used as an internal standard (*31, 45*). Razzazi-Fazeli *et al.* (*26*) used DEX, a synthetic glucocorticoid. This was employed as an internal standard to compensate for variability during the LC–APCI–MS analysis of urine samples. Although the retention time of the internal standard was very different from that of the tested type-B trichothecenes, the use of the internal standard allowed compensation for the drift of LC–MS response due to the deterioration of the APCI source, the cone and the focusing hexapoles. To take specific matrix effects into account, matrix matched calibration must be performed when no internal standard is available (*29*). A higher difference of the slope of the linear regression generally indicates effects such as suppression or enhancement of the signal. Sample extract dilution compensates most effects successfully; nevertheless for samples close to the limit of quantification only standard addition could verify the quantified concentration. Enhanced matrix effects, such as observed in soybean products by Biselli *et al.* (*29*) (Figure 2), require appropriate internal standards.

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Quadrupole Mass Analyzers

Quadrupoles have been most reported mass analyzers in the literature. Typically for both MS and MS/MS methods, optimization of the instrument parameters is performed by direct infusion of standard solutions through the ionization source while incrementally changing various parameters on the instrument.

Razzazi-Fazeli *et al.* (*26, 32, 43*) have conducted extensive research on trichothecenes using single quadrupole (MS) analysis. Selected Ion Monitoring (SIM) mode was used by selecting the molecule-related ions, associated adducts or the main fragments. Quantitation of the trichothecenes could be conducted using fragment ions. However, at low and high voltages, the S/N ratios of the Total Ion Current (TIC) deteriorated and therefore an optimum cone voltage must be evaluated. The intensive fragmentation of selected mycotoxins has the advantage of a diagnostic fingerprint, thus allowing identification of these compounds in the matrix.

Included in this research was a study into the effect of the cone voltage on the behavior of trichothecenes in the MS instrument. It was shown that by applying a potential difference between the sampling cone and the skimmer in the intermediate pressure region, Collision Induced Dissociation (CID) occurred in this region. When the cone voltage was increased, the kinetic energies of the ions also increased. It thus follows that higher amounts of energy are transferred into the internal energy of the analyte ions leading to more intense fragmentation. The intensity of the deprotonated molecules $[M-H]^-$ and the associated adducts decreases by increasing the cone voltage. Even at low cone voltages, when using a vaporizing temperature of 400 °C, the mass spectra of the compounds consisted of fragments and $[M-H]^-$ ions. Adduct ions of tested substances could be observed, which indicated the addition of two water molecules $[M–H+2H_2O]^-$ (*m/z* 331 for DON and *m/z* 347 for NIV). This indicates that the cone voltage plays a major role in the fragmentation processes of molecules. At cone voltages higher than 30 V, only fragment ions could be observed in the spectra of NIV and DON. For both molecules, the loss of 30 mass units was observed, giving ions of *m/z* 265 (for DON) and *m/z* 281 (for NIV). This was initially reported as cleavage of the epoxide ring, however later studies involving DOM, which is the de-epoxide form of DON, also showed a loss of 30. This indicated the cleavage of the C_{15} group as well as the epoxide ring (*48*). The other fragment ion observed for NIV occurs due to loss of a further two water molecules at *m/z* 245 $[M–H–CH_2O–2H_2O]^-$. For deoxynivalenol, a fragment at *m/z* 249 could be observed, which indicated the further loss of methane $[M–H–CH_2O–CH_4]^-$.

Razzazi-Fazeli *et al.* (*32*) also found that the mass spectra of T-2, MAS, and acetyl T-2 toxin (AcT-2 toxin) exhibited intensive fragmentation, even at low cone voltages. The mass spectra of T-2 contained a relatively intense signal due to the ammonium adduct $[M+NH_4]^+$, the $[M+H]^+$ ion and a fragment ion at *m/z* 365 $[M+H-(CH_3)_2CHCH_2COOH]^+$, due to the loss of the isovaleryl side chain (*32*). For AcT-2 toxin, an additional loss of isovaleryl (101 mu) was reported. The spectra of DAS and neosolaniol (NEO) were also reported in this study. DAS and NEO shared similar spectra showing ions such as $[M+H-CH_3COOH]^+$, $[M+H-H_2O]^+$ and a weak $[M+H]^+$, however for NEO, additional ions of $[M+H-H_2O-CH_3COOH]^+$ were observed.

Razzazi-Fazeli *et al.* (*26*) reported the spectra of 3-AcDON and 15-AcDON to be very similar but observed differences in the abundances of the product ions. The molecular ions of both compounds were observed, also the fragment *m/z* 291 was observed in both spectra but the 3-AcDON spectrum and showed higher intensity than 15-AcDON. On the other hand, in the case of 15-AcDON, relatively intensive fragment of *m/z* 277 as $[M-H-CH_3COOH]^-$ and *m/z* 295 as $[M-H-42]^-$ were reported. However, none of these were observed with an adequate intensity in the spectrum of 3AcDON. Berger *et al.* (*31*) also reported similar fragmentation ions for 3-AcDON using MS/MS in positive ion mode with QIT MS.

Lagana *et al.* (*35*) reported that the main disadvantage of single quadrupole MS analysis was the lack of possibilities for analyte confirmation due to the poor structural information that could be obtained. However, with LC-MS/MS instruments more structural information can be obtained and better analyte confirmation can be achieved. QqQ instruments can also yield better detection limits than single quadrupole instruments. These improved S/N ratios are due to the additional selectivity of the second MS step. Type-B trichothecenes showed specific fragmentation in negative ion mode. By selecting the $[M-H]^-$ ion of each compound as precursor ions for CID fragmentation, it was shown that a characteristic ion of $[M-H-30]^-$ could be produced for each trichothecene (*35*).

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The behavior of type-A trichothecenes in MS/MS experiments has also been investigated. Biselli *et al.* (*29*) observed four fragmentation reactions for T-2. Fragment ions at *m/z* 386 (loss of isovaleryl), *m/z* 326 and 266 (loss of acetate) and *m/z* 245 (structure unknown) were detected. After considering the levels of interference and the magnitude of detector response the 386 and 245 ions were selected for Multiple Reaction Monitoring (MRM) analysis. To cover possible shifts in the ratio between the $[M+H]^+$ and the $[M+Na]^+$ molecular ions, the linearity of T-2 response was investigated in a real commodity sample. The results of a four level calibration in whole wheat flour showed high linearity with a correlation coefficient of 0.993. Therefore, it was concluded that sodium adducts of type-A trichothecenes were acceptable precursor ions for fragmentation to produce further product ions.

Cavaliere *et al.* (*36*) maintained that sensitive MRM detection of trichothecenes required the presence of NH_4^+ ions in the mobile phase. Type-A trichothecenes form abundant adducts with ammonium and alkali metal ions which are in the LC grade solvents as impurities. Because sodiated adducts are often difficult to fragment Cavaliere *et al.* (*36*) used ammonia to promote the production of the $[M+NH_4]^+$ ion with respect to the $[M+Na]^+$ ion. This is advantageous since $[M+NH_4]^+$ ions yield fragmentation patterns which are more useful than that of sodiated adducts for MRM analysis. The fragments of sodiated precursor ions were clearly visible in the CID spectra, but the sodium ion itself was the most abundant of these fragments. In contrast the fragmentation of the ammonium adducts led to the loss of ammonia and subsequent neutral losses such as 18 Da (water from OH groups), 30 Da (formaldehyde from the epoxide ring), 60 Da (acetic acid from acetyl groups) and 101 Da (isovaleric acid from isovaleryl groups). The most intense of these transitions were selected for MRM analysis (Figure 5).

Cavaliere *et al.* (*36*) also investigated the behavior of type-B trichothecenes, initially using negative ion mode in the presence of acetate or formate. The $[M-H]^-$ ions of the type-B trichothecenes were not detected. However, as for many other polar compounds without acid or basic groups, type-B trichothecenes formed adducts with these anions. DON and NIV gave very intense $[M+COO]^-$ adducts, although when fragmented the formate ion predominated in the CID spectra and other less abundant fragments were available for MRM analysis. In contrast to this, 3-AcDON gave an $[M+H]^+$ ion about three times more abundant than the corresponding $[M+HCOO]^-$ and the signals from 15-AcDON were comparable in negative and positive ionization modes. The $[M+H]^+$ ion of both of these compounds was selected since better sensitivity was achieved in MRM mode. Despite the fact that 3-AcDON and 15-AcDON co-elute, the two compounds could be distinguished on the basis of their fragmentation. The two compounds can be distinguished in MRM mode by selecting different transitions (*30, 36*). It was also reported that among the type-B trichothecenes, the only one to form an $[M+NH_4]^+$ ion was Fus-X. The formation of this ion was probably due to the acetyl side chain at C_4, *syn* with respect to the epoxide ring. It was

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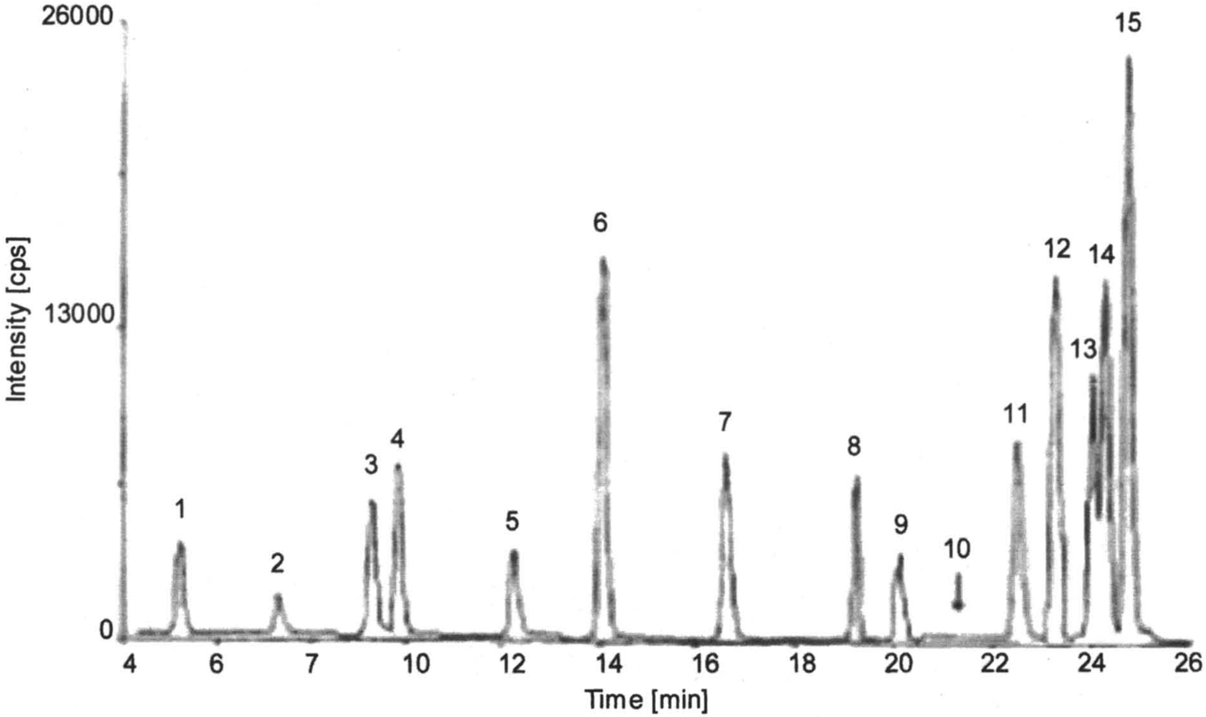

Figure 5. LC-MS/MS analysis of 16 mycotoxins, including 10 trichothecenes, using MRM by selecting the most abundant precursor and associated product ion transitions.1, NIV; 2, DON; 3, Fus-X; 4, Neo; 5, 3-AcDON and 15-AcDON; 6, MAS; 7, DAS; 8, Fumonisin B_1; 9, HT-2; 10, Fumonisin B_3; 11, T-2; 12, Fumonisin B_2; 13, a-zearalenol; 14, ZAN; 15, ZON. [Reprinted from Cavaliere et al. *(36), with permission. Copyright (2005) John Wiley & Sons Ltd.]*

also shown that the fragmentation of this ion was very similar to that of the type-A trichothecenes (*36*).

Quadrupole Ion Trap (QIT) Mass Analyzer

QIT MS instruments have been used in mycotoxin analysis for both quantitative and qualitative purposes. While QIT MS is quite capable of providing good quantitative data, its real power lies in the ability to conduct MS^n studies. These experiments can yield excellent structural data of analytes. In MS^n mode, the QIT collects ions at a defined *m/z* ratio while ejecting almost all other ions. An excitation signal is applied to the poles of the ion trap to excite selected ions, which collide with the collision gas and fragment. The subsequent fragmentation ions are expelled from the trap and acquired as the spectrum.

Berger *et al.* (*31*) conducted an in-depth investigation of the fragmentation of trichothecenes using QIT MS. In this study, molecule-related ions were exposed to CID fragmentation which led to the loss of neutral molecules. These neutral losses originated mainly from the oxygenated substituents (except the carbonyl oxygen) and the epoxide ring. It was also shown that when comparing type-A and type-B trichothecenes, the type B trichothecenes easily lose the C_{15} side chain, resulting in a quinoic structure followed by rearrangement into a suitable aromatic alcohol. The study also identified the neutral losses from the compounds as 18 Da (H_2O from OH groups), 28 Da (CO from the epoxide ring), 30 Da (CH_2O from the epoxide ring or from the OH group at the C15 position for type-B trichothecenes), 42 Da (CH_2CO from acetyl), 60 Da (CH_3COOH from acetyl) and 102 Da ($(CH_3)_2CHCH_2COOH$ from isovaleryl). The data from MS^n and ion adduct studies allowed the development of a structural elucidation scheme for trichothecenes (Figure 6). This scheme facilitated the identification of trichothecenes in an LC-MS/MS analysis as well as identifying unknown type-A or type-B trichothecenes (*31*).

Young *et al.* (*49*) used QIT MS to investigate the degradation products of trichothecenes following reaction with ozone. The mass spectral data acquired during this study showed that the degradation products resulted from the net addition of two oxygen atoms to the molecule. When the CID spectra of the major product ions from each of the trichothecenes (which was offset by 32 mass units) was compared with the CID spectra of the starting compounds, there was virtually complete matching of the fragmentation patterns. All of the MS data showed that, aside from the change in oxidation state at C_{9-10}, the remainder of the molecule was left intact.

Using QIT MS, Plattner *et al.* (*34*) showed that DON could be detected in grain and food products without sample clean-up. In this work the detection limits were reported as approximately 2 ppm, but following sample clean-up the LOD's were 0.2 ppm. These results were obtained by using the QIT in LC-MS mode. Later, it was shown that by operating the instrument in LC-MS/MS mode, detection limits could be dramatically improved (>50 fold) (*46*).

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In MS/MS mode, only fragments arising from the selected precursor ion are detected and most of the chemical noise from other signals in the matrix are eliminated, thus improving the S/N ratio. Tuomi *et al.* (*27*) used the sodiated molecule-related ions as precursor ions for MS/MS analysis using QIT MS. They optimized the production of $[M+Na]^+$ ions relative to the sodiated dimers and trimers, thus improving the LOD's.

Royer *et al.* (*45*) also conducted LC-MS/MS analysis of trichothecenes. To allow unambiguous confirmation of the toxins two transitions were monitored for each trichothecene, one qualitative and one confirmatory, and the area ratio of the two traces for each analyte were obtained. Confirmation of the trichothecenes was accepted when three criteria were met. Firstly; the two transition reactions need to co-elute at the same retention time, secondly; this retention time was observed within ± 2.5% of the standard, and finally; the transition reactions ratio for the confirmation transition versus the quantitation transitions needed to be within a predefined coefficient of variation.

Time-of-Flight (TOF) Mass Analyzer

TOF analyzers have only recently been applied to the analysis of trichothecenes. Previously, LC-TOF-MS has only been applied for confirmatory analysis mainly because of the limitations, such as a narrow dynamic linear range obtained with this type of mass spectrometer. However, recent developments in this technology have allowed these drawbacks to be overcome.

It has been shown that some problems can be encountered when analyzing multiple compounds using LC-MS or LC-MS/MS. In LC-MS, when scanning a wide mass range for a number of compounds with different molecular weights the scanning demands made on the instrument result in a loss of sensitivity. Conversely when using the MRM mode to boost sensitivity, poor analyte confirmation may result since full mass spectral data are not acquired. In contrast to these drawbacks, LC-TOF-MS has the ability to detect a wide mass range without losing significant sensitivity. TOF instruments also provide enhanced full mass range spectra sensitivity and accuracy due to their higher mass resolution. This also makes it possible to distinguish among isobaric ions and target ions and increases confidence in the identification of analytes by giving high mass accuracy and an estimate of the elemental composition of each ion. Furthermore, LC-TOF-MS has the ability to perform quantitation on any ion detected in the scan range of the instrument.

Nielsen *et al.* (*49*) undertook a High Throughput Screening (HTS) program for the detection of 474 fungal metabolites in culture extracts including trichothecenes. They reported that because of the labile properties of the trichothecenes, they must be analyzed at lower cone voltages to yield higher abundance of high mass ions other than $[M+Na]^+$ ion, which dominate at higher cone voltages. This lability had also been reported previously (*32, 43*). The high

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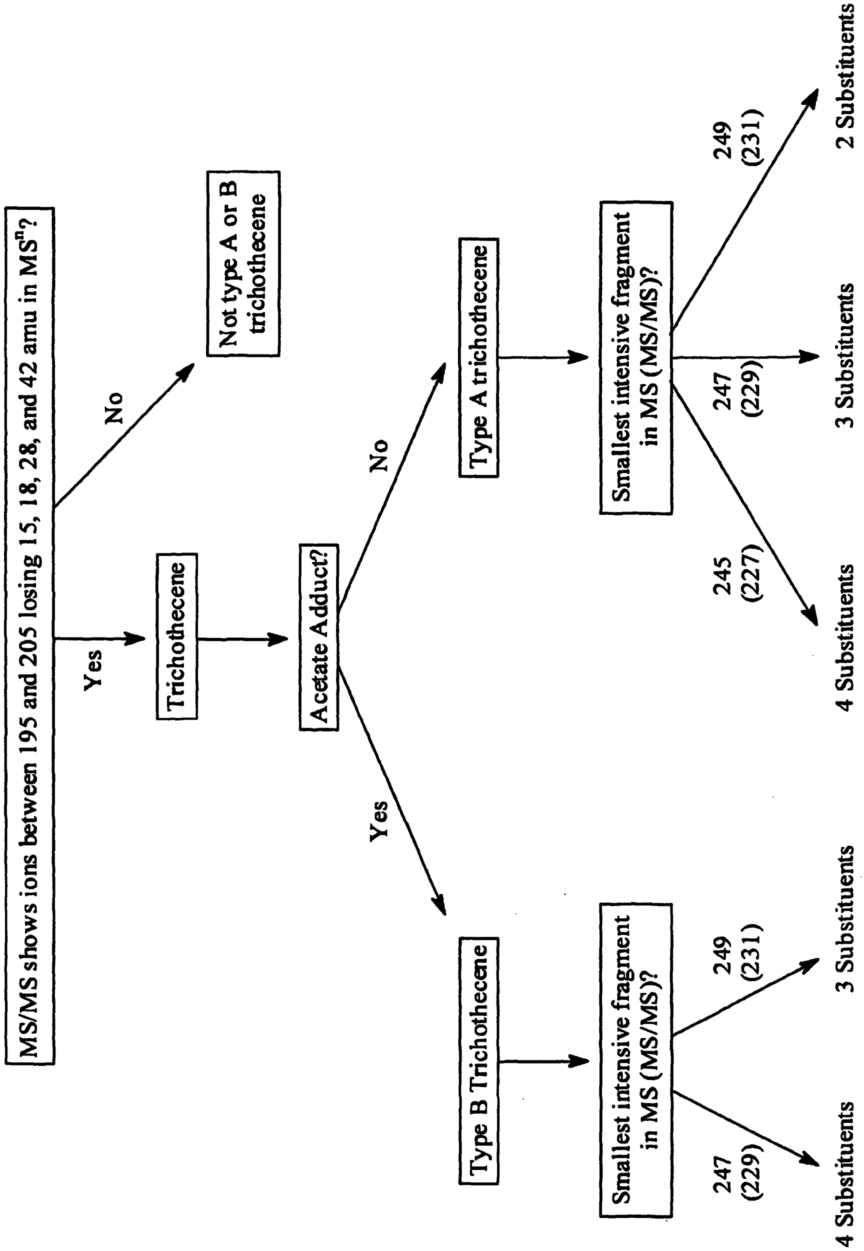
MS/MS shows ions between 195 and 205 losing 15, 18, 28, and 42 amu in MS^n?
Yes
No
Trichothecene
Not type A or B trichothecene
Acetate Adduct?
Yes
No
Type B Trichothecene
Type A trichothecene
Smallest intensive fragment in MS (MS/MS)?
Smallest intensive fragment in MS (MS/MS)?
247 (229)
249 (231)
245 (227)
247 (229)
249 (231)
4 Substituents
3 Substituents
4 Substituents
3 Substituents
2 Substituents

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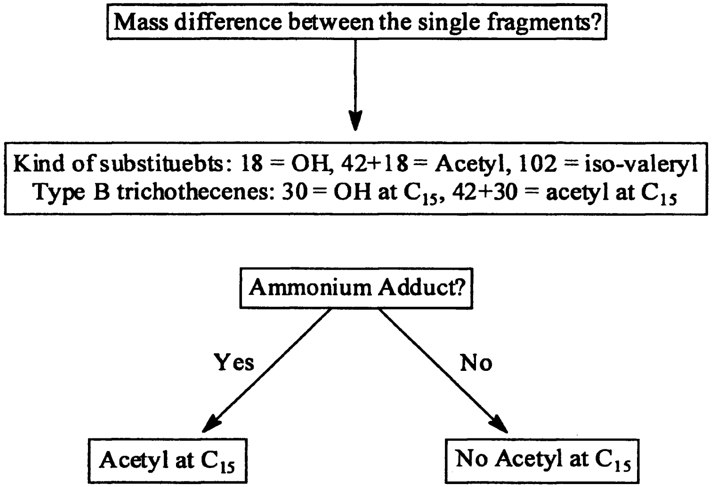
Mass difference between the single fragments?
Kind of substituebts: 18 = OH, 42+18 = Acetyl, 102 = iso-valeryl
Type B trichothecenes: 30 = OH at C_{15}, 42+30 = acetyl at C_{15}
Ammonium Adduct?
Yes
No
Acetyl at C_{15}
No Acetyl at C_{15}

Figure 6. Identification scheme for the structural elucidation of trichothecenes using QIT MS. [Adapted from Berger et al. *(31) with permission. Copyright (1999) American Chemical Society].*

mass accuracy for the metabolites was recorded in the range of 3-4 ppm. However, it was highlighted in this study that the best high mass accuracies will be obtained at the front or tail of the chromatographic peak. Determining high mass accuracy at the peak maximum may not be advisable since the ion counts may be too high (*49*).

Tanaka *et al.* (*50*) underlined the advantage of high mass accuracy in a recent study of the determination of trichothecenes using LC-TOF-MS. Of particular note was the determination of NIV. The ion used for the determination was identified as $[M+NH_4-H_2O]^+$ but this identification would not have been possible with a low resolution mass spectrometer since the mass difference between this ion and $[M]^+$ is only 0.02. The mass errors of all mycotoxins in the study were in the range of -2.49 to 2.12 ppm. These accurate mass measurements greatly increased the confidence in the correct assignment of the compounds of interest.

It has been shown that mass accuracy and signal intensity of the analyte can be affected by compounds from the matrix. Tanaka *et al.* (*50*) investigated these matrix effects by comparing exact mass chromatograms and the accurate mass of base peak ions obtained from standard solutions in pure water and in the matrix extract. It was reported that the mass error of each mycotoxin in all the foodstuffs used was within acceptable limits (± 5 ppm); it was also shown that no significant reduction and enhancement of the TOF-MS responses were observed.

The high mass accuracy data obtained from TOF instruments can also be used for structural confirmation of neutral losses involved in CID experiments. The authors have conducted NanoSpray MS/MS experiments on the trichothecene neosolaniol (NEO) using a hybrid quadrupole TOF (QqTOF) mass spectrometer to produce a high mass accuracy MS/MS spectrum (Figure 7). In this spectrum the peak at *m/z* 400 represents the ammoniated adduct ion.

The peak *m/z* 365 represents the $[M+H-H_2O]^+$ ion which is produced by the double loss of ammonia and water, $[M+NH_4-NH_3-H_2O]^+$. The $[M+H-H_2O]^+$ ion can then undergo successive CH_3COOH losses to produce *m/z* 305, *m/z* 245, and *m/z* 185. The *m/z* 275 peak is formed by CH_2O loss from *m/z* 305. It can also be shown using QIT MS that different fragmentation pathways can produce the same product ions, Thus, the *m/z* 245 ion can be represented as $[M+H-H_2O-CH_3COOH-CH_3COOH]^+$ or as $[M+H-H_2O-CH_2O-CH_3COOH-CH_2O]^+$. This ability to recognize multiple fragmentation routes and the ability to provide high mass accuracy data shows that TOF instruments can complement the data produced in MS^n studies with QIT MS instruments, enabling the fragmentation pathways of compounds can be studied.

Conclusion

LC-MS has been adopted worldwide for the detection of trichothecenes. ELISA and TLC methods are still used regularly for screening but cannot match

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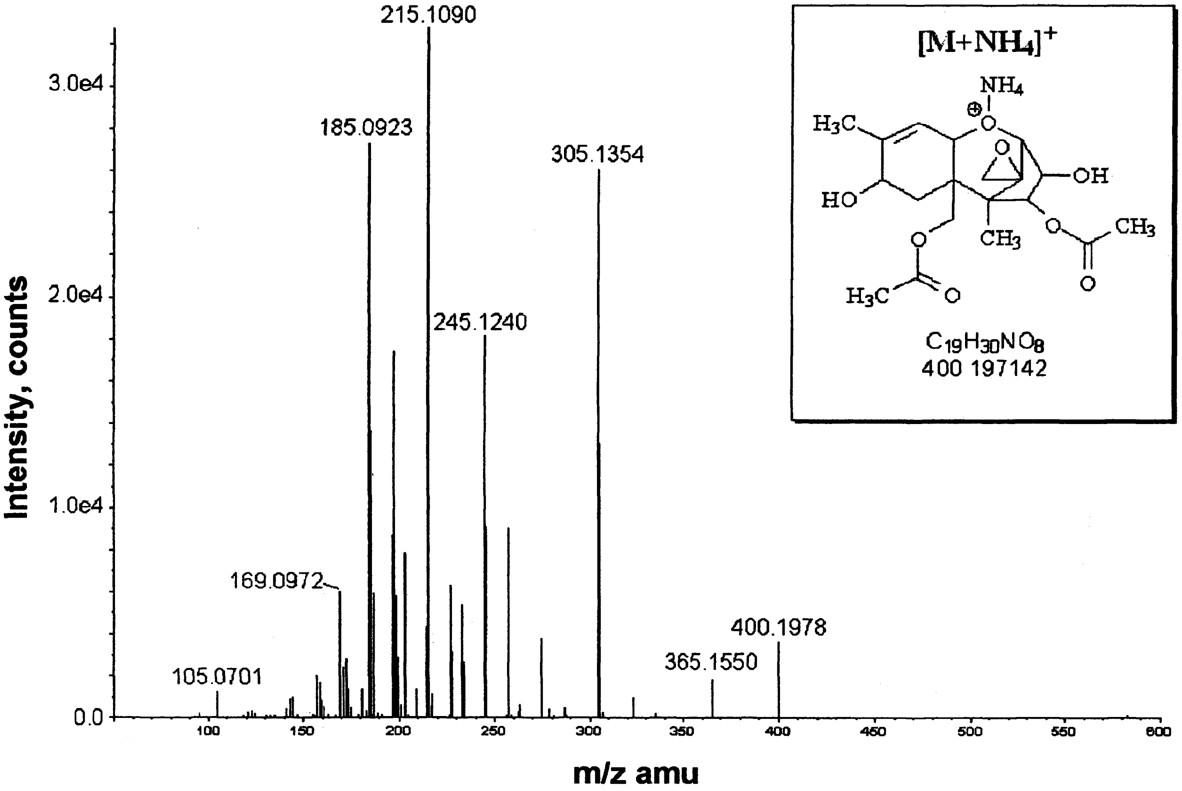

Figure 7. MS/MS spectrum of the neosolaniol ammonium molecular ion obtained using nanospray ionization on a hybrid Quadrupole-Time of Flight Mass Spectrometer(QqTOF MS).

the ability of LC-MS to provide high quality confirmatory and quantitative data. As a result LC-MS methods have become the standard in trichothecene analysis since they eliminate the need for derivatization and the possibility of false positives. With the continued development of mass spectrometry the sensitivity and selectivity of these methods continue to improve ensuring LC-MS will remain the benchmark for the future.

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Chapter 12

Detection of Relevant Mycotoxins in Wheat Beer and Sake by LC–MS/MS Using Prototype Immunoaffinity Column Clean-Up: A Preliminary Study

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All of 12 relevant mycotoxins, namely deoxynivalenol (DON), aflatoxins B_1, B_2, G_1 and G_2, ochratoxin A (OTA), fumonisins B_1, B_2 and B_3, T-2 toxin (T-2), HT-2 toxin (HT-2), and zearalenone (ZON) present in complex wheat beer and sake matrices were extracted using multifunctional mixed bed immunoaffinity columns. They were detected using a LC-MS/MS method that achieved good separation and detection. Recoveries of all the toxins from the immunoaffinity columns ranged from 64-127%. The LC separation was performed using a reverse phase C18 column. MS measurements were done in both the positive and negative ESI modes. Multiple reaction monitoring (MRM) was carried out for each compound.

In Food Contaminants; Siantar, D., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2008.

Mycotoxins are toxic metabolites produced in food and feed by field and storage fungi. The occurrence of mycotoxins in a variety of foods has become a great concern as they are often associated with acute and chronic toxicity in humans and animals. The most important mycotoxins are aflatoxins, ochratoxin A (OTA), trichothecenes, fumonisins, and zearalenone (ZON). Because of their acute and chronic toxicity, guidelines and tolerance levels have been set for food and feed worldwide. Contamination of fermented alcoholic beverages such as wheat beer or sake is possible if contaminated wheat or rice are used in the manufacturing process. Several mycotoxins have been found in beers of different countries (*1*). It is therefore very important to monitor for mycotoxins in beverages before they are released for public use.

Several analytical methods such as GC-MS and LC with UV, fluorescence, and MS detection have been used for the analysis of mycotoxins (*2-4*). LC-MS/MS has broad applicability and is highly selective. Matrix effects are generally observed while analyzing the mycotoxins present in complex matrices. Sample preparation that helps minimize the ion suppression or enhancement is required prior to the LC-MS/MS analysis. Immunoaffinity clean-up has been extensively used for the extraction of mycotoxins from complex matrices and the extracted compounds were generally analysed by LC-fluorescence or LC-UV and LC-MS analysis (*5-9*). Presently available immunoaffinity catridges extract one, two or three groups of mycotoxins at a time. In the present study we have evaluated two different mixed bed multifunctional cartridges, one that retains four mycotoxins and the other that retains 12 mycotoxins simultaneously. Determination was by an LC-MS/MS method.

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Materials and Methods

Materials

Aflatoxins B_1, B_2, G_1 and G_2, deoxynivalenol, T-2, HT-2, ochratoxin A, zearalenone, zearalanone (internal standard), fumonisins B_1 and B_2 standards were purchased from Sigma–Aldrich Co, St. Louis, MO. Fumonisin B_3 was from PROMEC, the Medical Research Council in Tygerberg, South Africa. Acetic acid (analytical-reagent grade) and HPLC grade methanol were purchased from Fisher Chemicals (Boston, MA). Water (conductivity of 18 $M\Omega^{-1}$) was purified by a Picotech Hydro UltraPure Water System (Garfield, NJ, USA).

Instruments

A Varian 320-MS LC-MS/MS equipped with an ESI source coupled to Varian Prostar 210™ solvent delivery system and Varian Prostar 430™ autosampler were used for this work. Chromatographic separation was achieved on a 150 mm x 3.0 mm id RP-C18 column. MRM and atmospheric ionization (API) conditions given in our previously published multicomponent LC-MS/MS method (*10*) were used to analyze the wheat beer and sake samples that were cleaned-up using the prototype multifunctional immunoaffinity columns. Details of the LC-MS/MS method are as follows. MRM conditions are given in Table 1.

LC Conditions

Column: Polaris C-18A 5μM, 150 mm x 3.0 mm id
(Varian part No A2000150x030)
Buffer A: 5 mM ammonium acetate, 1% CH_3COOH in 10% methanol
Buffer B: 5 mM ammonium acetate, 1% CH_3COOH in 100% methanol
LC Gradient: 15% to 60% buffer B in 40 min at 0.3 mL/min

API Conditions

Ionization Mode: ESI (positive and negative)
Collision Gas: Argon, 1.8 mTorr
API Drying Gas: 30 psi at 250 °C
API Nebulizing Gas: 50 psi
Needle: 4500V
Detector: 1900V

Sample Preparation

Two immunoaffinity columns, one provided by VICAM and the other by R-Biopharm Rhône, were used for this study. VICAM's "all-in-one" prototype immunoaffinity column has antibodies for the simultaneous purification of 12 mycotoxins namely DON, ZON, T-2, HT-2, aflatoxins B_1, B_2, G_1, G_2, fumonisins B_1, B_2, B_3, and ochratoxin A. R-Biopharm Rhône's prototype immunoaffinity column has antibodies for DON, ZON, T-2, and HT-2. Entire sample preparation was performed using Zymark's automated SPE work station. Stock solutions of individual mycotoxin standards were prepared by mixing pure standards in methanol and storing at 4 °C until use. These standards were used to make mixed standard solutions of all 12 analytes. Dealcoholized wheat beer and sake samples were spiked with the mixed toxin standard at known concentrations. The internal standard zearalanone spike concentration was 70 ng/g. The above samples were degassed by ultrasonication for 15 min and filtered through Whatman No.1 filter paper. Three mL of these samples were mixed with three mL of 10% phosphate buffered saline (PBS) aqueous solution.

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Table1. Multitoxin method - MRM parameters

Mycotoxin	*Precursor Ion (m/z)*	*Product Ion 1 (m/z)*	*Collision Energy 1 (V)*	*Product Ion 2 (m/z)*	*Collision Energy 2 (V)*	*Dwell (sec)*
Deoxynivalenol	355.1	59.2	25.0	265.0	11.5	0.3
Aflatoxin G_2	331.0	245.0	16.5	275.0	11.5	0.3
Aflatoxin G_1	329.0	283.0	25.5	243.0	25.5	0.3
Aflatoxin B_2	315.0	287.0	21.5	259.0	10.0	0.3
Aflatoxin B_1	313.0	245.0	16.5	241.0	22.0	0.3
Fumonisin B_1	720.9	157.0	33.0	563.0	24.0	0.3
Fumonisin B_2	706.3	332.2	33.0	318.1	40.0	0.3
Fumonisin B_3	704.6	546.0	33.0	159.0	33.0	0.3
T-2	484.0	305.0	18.5	117.0	13.5	0.3
OTA	403.9	238.9	25.0	357.9	25.0	0.3
Zearalanone	319.0	136.0	26.5	187.0	28.5	0.3
ZON	317.0	185.0	24.5	187.0	18.5	0.3
HT-2	447.0	265.0	11.5	345.0	13.5	0.3

The solution was then passed through VICAM's or R-Biopharm's prototype immunoaffinity column. The column was washed with 5 mL of deionized water. Mycotoxins were eluted using 3 mL methanol. This solution was further dried under nitrogen, reconstituted to a known volume with LC buffer and then analyzed by LC-MS/MS.

Results and Discussion

Wheat beer or sake spiked with mixed toxin standard at medium level (DON, ZON, T-2, HT-2, fumonisins B_1, B_2, and B_3 at 350 ng/g, aflatoxins B_1, B_2, G_1 and G_2 at 35 ng/g, and ochratoxin A at 175 ng/g) were extracted using the VICAM mixed bed multifunctional immunoaffinity cartridge that simultaneously retains all the 12 mycotoxins. Mixed toxin standard spiked in 10% PBS (with no alcoholic beverage matrix) loaded into the multifunctional column using the above procedure was used as the control sample. Unspiked sake or wheat beer passed through the mixed bed immunoaffinity column served as the blank. The eluate from the cartridge was collected and analyzed using an LC-MS/MS method that achieved good separation and detection for all the 12 mycotoxins. The LC-MS/MS total ion chromatogram (TIC) of the multi-toxin spiked sake is given in Figure 1; good separation and peak shapes were obtained for all 12 mycotoxins. The MRM chromatogram of the 12 mycotoxins with zearalanone (ZAN) as an internal standard is given in Figure 2.

Triplicate recoveries of the mycotoxins were calculated (Table 2). The recoveries were acceptable for all 12 mycotoxins indicating the high specificity of monoclonal antibodies and ease of use of immunoaffinity column clean-up. The RSD's were very low indicating excellent selectivity and robust clean-up of the immunoaffinity column for mycotoxins from wheat beer and sake.

In the next step, triplicate recoveries of the four mycotoxins namely, DON, ZON, T-2 and HT-2 at low spike concentration (35 ng/g) extracted from a complex wheat beer matrix using R-Biopharm and VICAM's mixed bed columns were compared (Table 3). The recoveries obtained from both the columns were above 95% and were comparable. Well-resolved peak shapes were obtained indicating the suitability of immunoaffinity column clean-up for quantitation by LC-MS/MS at low levels (Figure 2). Recoveries met the EU requirements for the recovery rates of mycotoxins from complex matrices (2005/38/EG) (11). In the guideline 2005/38/EG, the recovery limits are given as follows: 60 to 120% for DON/ZON and 60 to 130% for T-2/HT-2.

Recently, recovery rates of 100 ± 2.1 to 3.3% were reported using RP C18 columns for ZON, α-zearalenol and β-zearalenol from 23 beer samples (12). Dall' Asta and co-workers demonstrated that sample clean-up may be omitted for the direct injection of wine samples on LC-MS and LC-fluorescence systems (13). Although, good recoveries were achieved, it remains doubtful that a similar

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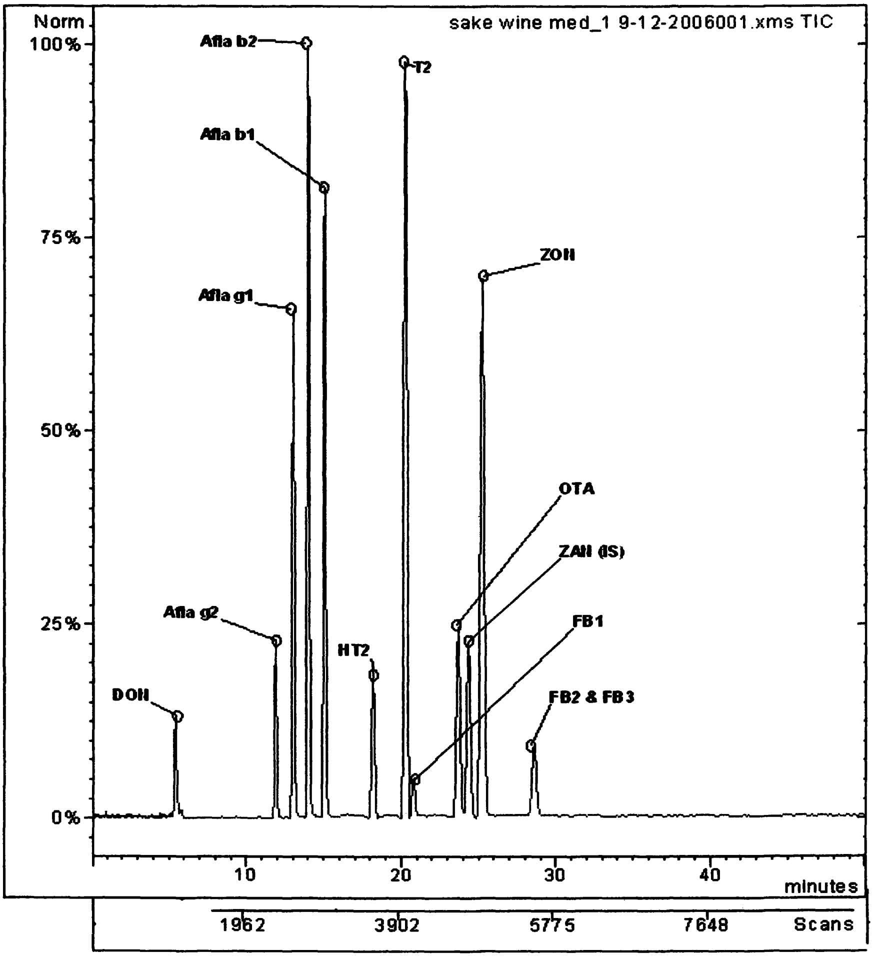

Figure 1. Total ion chromatogram (TIC) of the 12 mycotoxins purified from sake using VICAM'S mixed bed multifunctional immunoaffinity column. [NOTE: Aflatoxins and Fumonisins are abbreviated as Afla and FB]

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Table 2. Mycotoxin Recoveries Obtained Using a Mixed Bed Multifunctional Immunoaffinity Column (n=3)

	VICAM Recovery % ± RSD% at medium spike level	
Mycotoxin	*Wheat beer*	*Sake*
DON	98.1 ± 1.4	92.8 ± 1.5
Aflatoxin B_1	100.0 ± 3.2	93.1 ± 1.7
Aflatoxin B_2	92.5 ± 2.3	81.7 ± 0.7
Aflatoxin G_1	92.5 ± 1.2	93.1 ± 3.1
Aflatoxin G_2	91.7 ± 2.1	81.8 ± 5.3
T-2	99.9 ± 1.7	100.7 ± 1.2
HT-2	64.0 ± 3.9	74.0 ± 1.0
OTA	102.4 ± 3.9	111 ± 0.8
Fumonisin B_1	107.0 ± 5.9	107.9 ± 2.0
Fumonisin B_2	107.8 ± 13.8	95.7 ± 6.5
Fumonisin B_3	116.4 ± 2.8	127.0 ± 6.9
ZON	101.6 ± 3.1	101.6 ± 4.3

Table 3. Comparison of VICAM's and R-Biopharm's Mixed Bed Multifunctional Immunoaffinity Columns for the Recovery of DON, ZON, T-2 and HT-2 (n=3)

	Recovery % ± RSD % in Wheat Beer at Low Spike Level for Four Mycotoxins	
Mycotoxin	*VICAM*	*R-Biopharm*
DON	95.5 ± 2.3	101.8 ± 2.8
ZON	115.8 ± 1.3	113 ± 1.0
T-2	110 ± 4.5	96 ± 1.4
HT-2	107.6 ± 1.39	101.9 ± 1.4

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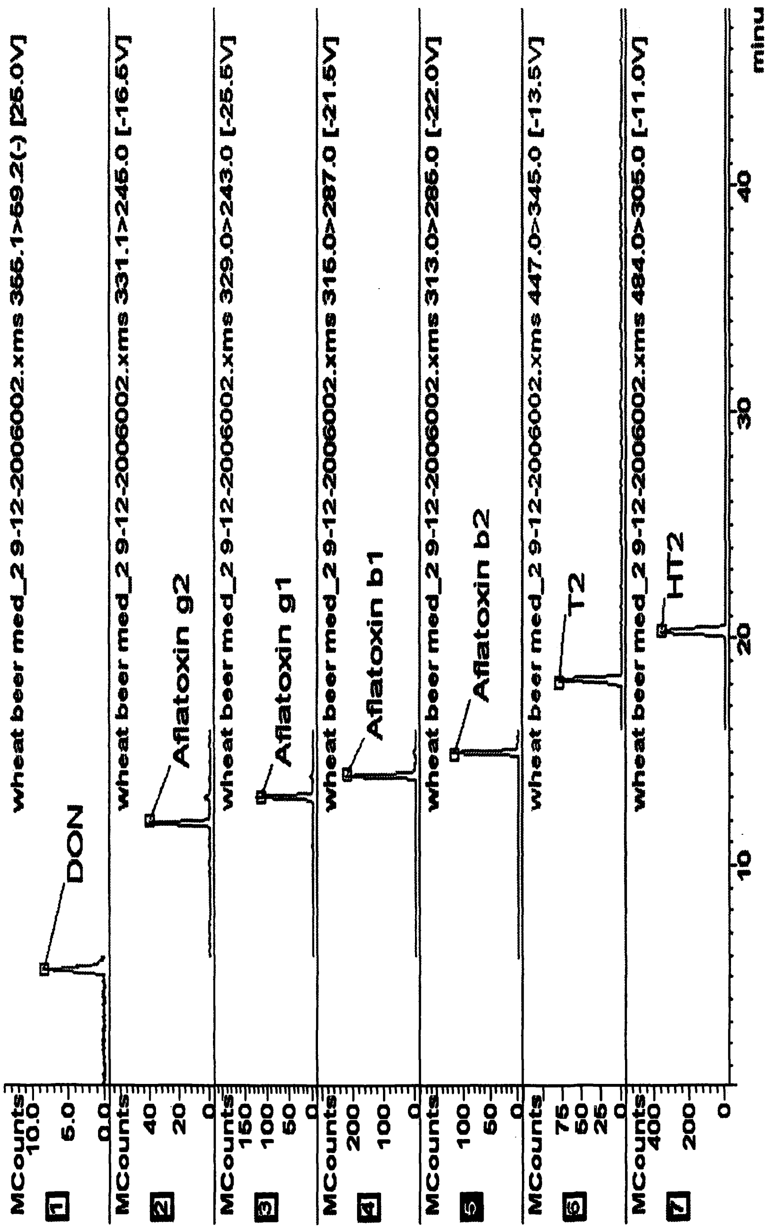

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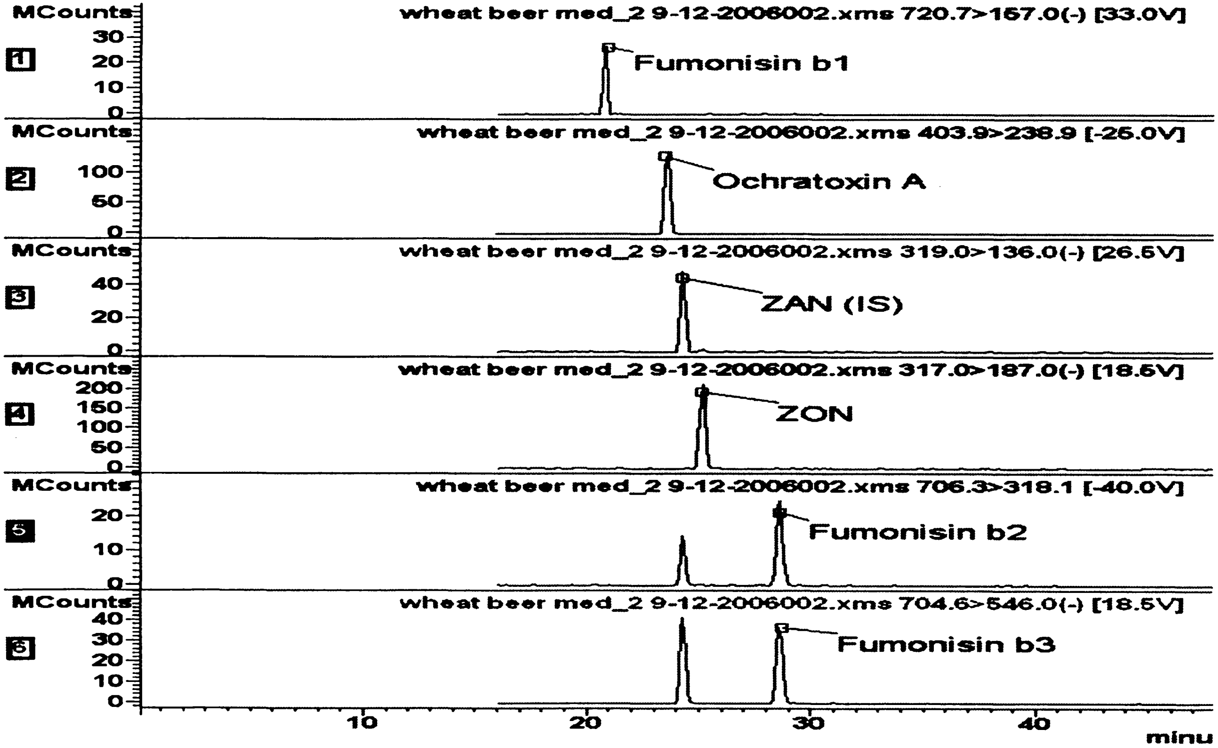

Figure 2. MRM chromatogram of the 12 mycotoxins and an internal standard pre-spiked in wheat beer at 350 ng/g and extracted using the VICAM cartridge.

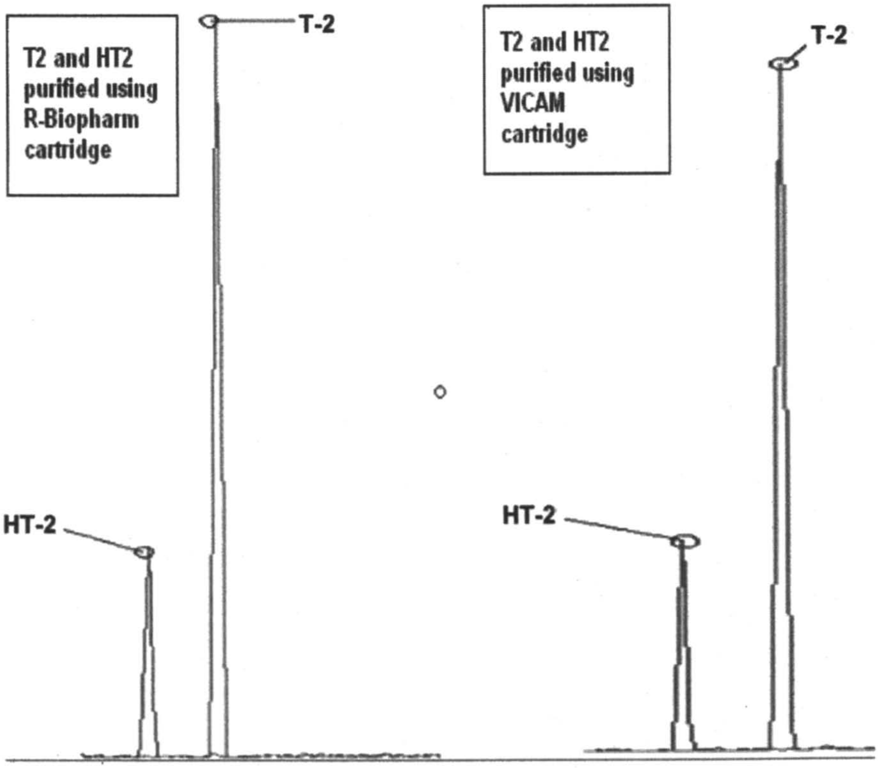

Figure 3. LC-MS/MS chromatograms of T-2 and HT-2 toxins extracted from wheat beer using VICAM and R-Biopharm's cartridges at 35 ng/g.

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method can be successfully applied without any matrix interferences especially at low mycotoxin concentrations. In such cases, immunoaffinity columns containing immobilized antibodies that selectively retain mycotoxins, that produce cleaner extracts with a minimum level of interfering matrix components, and that have excellent signal to noise ratios compared to polymeric SPE sorbent materials would be an ideal choice.

Conclusion

Recoveries of 12 mycotoxins from the the prototype mixed bed multifunctional immunoaffinity columns are all in acceptable ranges. Mixed bed immunoaffinity purification of mycotoxins coupled with LC-MS/MS analysis is an excellent solution to the simultaneous detection of several mycotoxins present in complex matrices.

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Chapter 13

Quantitation of Trichothecene Mycotoxins by Stable Isotope Dilution Assays

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Stable isotope dilution assays (SIDAs) for the simultaneous quantitation of type A and type B trichothecenes in cereal products were developed. Syntheses of [$^{13}C_4$]-diacetoxyscirpenol, [$^{13}C_2$]-monoacetoxyscirpenol, [$^{13}C_4$]-T-2 toxin, [$^{13}C_2$]-HT-2 toxin, [$^{13}C_2$]-3-acetyldeoxynivalenol, [$^{13}C_2$]-15-acetyldeoxynivalenol, and [$^{13}C_2$]-4-acetylnivalenol were accomplished by complete [$^{13}C_2$]-acetylation of T-2 triol, scirpentriol, deoxynivalenol, and nivalenol, respectively, followed by careful alkaline hydrolysis of the peracetylated compounds. Samples were spiked with the synthesized internal standards and purified on multifunctional columns. All trichothecenes under study were quantified simultaneously within one liquid chromatography (LC)-mass spectrometry (MS) run using single and tandem MS detection. The method revealed good sensitivity with low detection and quantification limits along with excellent recovery data and good precision in inter-assay studies. Food samples were analysed using the developed SIDA and showed substantial contamination of oat products with T-2 toxin and HT-2 toxin. Diacetoxyscirpenol was detected rarely and monoacetoxyscirpenol and 4-acetylnivalenol were not present in the analyzed samples. Further type B trichothecenes occurred more frequently and generally in higher concentrations than type A trichothecenes. The type B trichothecene deoxynivalenol was detectable in all cereal samples. Corn (maize) products did not only show the highest concentrations of deoxynivalenol, with values up to 300 µg/kg, but also distinct contamination with 15-acetyl-

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ACS Symposium Series; American Chemical Society: Washington, DC, 2008.

deoxynivalenol, whereas 3-acetyldeoxynivalenol was predominantly found in oat products.

Trichothecene mycotoxins are produced by various fungi of the genus *Fusarium,* which infect cereals during growth and cause a characteristic disease pattern called *Fusarium* head blight in wheat and *Gibberella* ear rot in corn.

These mycotoxins are tetracyclic sesquiterpenes bearing a spirocyclic epoxide moiety and are classified into four different types A, B, C, and D. Type A trichothecenes differ from those belonging to the type B by the absence of a carbonyl group at C-8 and hydroxylation at C-7. The type A group itself can be differentiated into two families namely the T-2 family with hydroxylation at C-8 and the scirpenol family, which is completely devoid of any functionalities at C-8. Structures of common trichothecenes are shown in Figure 1.

Due to the production of mycotoxins, infections of cereals with *Fusarium* present an acute hazard to the consumer. As trichothecenes are known to cause vomiting and hematoxic effects as well as immunosuppressive effects, a temporary tolerable daily intake (tTDI) value of 0.06 μg/kg bw/d for the sum of T-2 toxin and HT-2 toxin, as well as a tTDI of 1 μg/kg bw/d and 0.7 μg/kg bw/d for the type B trichothecenes deoxynivalenol and nivalenol, respectively, has been suggested by the Scientific Committee on Food (SCF) (*1*).

Therefore, trace concentrations of these compounds have to be controlled in foods, which requires sensitive and accurate methods for quantitation. Among the most reliable analytical methods, stable isotope dilution assays (SIDA) are becoming more common as reference methods to determine the "true" analytical value. In mycotoxin analysis, this concept already has been successfully applied to quantitation of patulin (*2*) and ochratoxin A (*3*).

In the past, trichothecenes have commonly been analyzed by gas chromatography with either flame-ionization detection (*4*), electron-capture detection (*5*), or mass spectrometric detection (*6*). Due to the polarity of the free hydroxyl moieties of trichothecenes, derivatization is necessary to afford volatile compounds for GC analysis. As this additional step in sample preparation is time consuming and may lead to increased analyte loss, direct analytical methods such as LC are generally preferred.

However, LC-UV methods, which today are routinely applied to the determination of type B trichothecenes, cannot be used for type A trichothecenes due to lack of a UV-absorbing moiety in the molecular structure. As a consequence, methods for the determination of trichothecenes have been developed using LC with mass spectrometric (MS) detection (*7*).

Due to losses during clean-up and ionization interferences of matrix compounds, the application of internal standards is often necessary during LC-MS. For trichothecenes, the use of structurally similar internal standards such as

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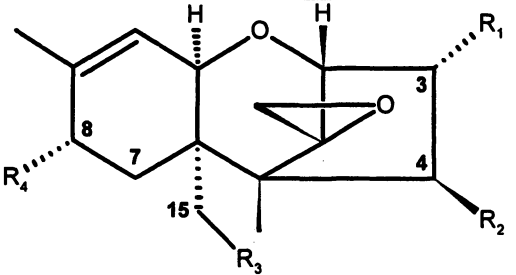
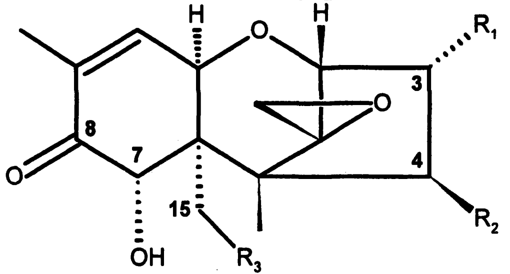
Type A trichothecenes:



	R_1	R_2	R_3	R_4
HT-2 toxin	OH	OH	OAc	$OCOC_4H_9$
T-2 toxin	OH	OAc	OAc	$OCOC_4H_9$
Monoacetoxyscirpenol	OH	OH	OAc	H
Diacetoxyscirpenol	OH	OAc	OAc	H

Type B trichothecenes:



	R_1	R_2	R_3
Deoxynivalenol	OH	H	OH
3-Acetyldeoxynivalenol	OAc	H	OH
15-Acetyldeoxynivalenol	OH	H	OAc
4-Acetylnivalenol	OH	OAc	OH

Figure 1. Structures of common trichothecenes.

deepoxy-deoxynivalenol, neosolaniol, and verrucarol (*7*) has been reported. Regarding the ideal structure for internal standards, there is general consensus that the use of isotopically labelled internal standards not only can compensate best for losses of analytes during sample preparation but also for any kind of matrix effect. Therefore, the aim of the present study was to synthesize stable isotopically labelled analogues of trichothecenes and to develop SIDAs for the simultaneous quantitation of these mycotoxins.

Materials and Methods

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Stable isotope labeled trichothecenes used as internal standards were synthesized by [$^{13}C_2$]-acetylation as described recently (*8, 9*). In brief, [$^{13}C_4$]-T-2 toxin and [$^{13}C_2$]-HT-2 toxin were obtained by alkaline hydrolysis of [$^{13}C_6$]-T-2 3,4,15 triacetate, which itself was prepared by complete acetylation of T-2 triol with [$^{13}C_4$]-acetic anhydride in dry pyridine. Analogously, [$^{13}C_2$]-monoacetoxyscirpenol ([$^{13}C_2$]-MAS) and [$^{13}C_4$]-diacetoxyscirpenol ([$^{13}C_4$]-DAS) were prepared from scirpentriol, [$^{13}C_2$]-4-acetylnivalenol ([$^{13}C_2$]-4-AcNIV) from nivalenol, and [$^{13}C_2$]-3-acetyldeoxynivalenol ([$^{13}C_2$]-3-AcDON) and [$^{13}C_2$]-15-acetyldeoxynivalenol ([$^{13}C_2$]-15-AcDON) from deoxynivalenol (DON). Identity and purity of all synthesized compounds were verified using NMR spectroscopy, LC-MS, and LC-MS/MS experiments before they were used as internal standards. [$^{13}C_{15}$]-Deoxynivalenol was obtained from Biopure Referenz-substanzen GmbH (Tulln, Austria).

Analysis of trichothecenes was performed using LC-MS. LC separation prior to MS was achieved on a Finnigan Surveyor Plus LC System (Thermo Electron Corp., Waltham, MA) using a 150 x 2 mm i.d., 4 µm Synergi Polar-RP column (Phenomenex, Aschaffenburg, Germany) as the stationary phase. The mobile phase consisted of variable mixtures of formic acid (0.1 %, solvent A) and acetonitrile acidified with formic acid (0.1 %, solvent B). A linear gradient was programmed starting with 5 % B, which was held for 5 min and then increased to 30 % B within 15 min. Immediately afterwards the concentration of B was raised to 60 % within 2 min and then increased more slowly to reach 100 % B 30 min after injection. 100 % B was held for 10 min before returning to initial conditions. The subsequent equilibration time between two runs was set to 15 min. Flow rate was 0.2 mL/min and the injection volume was 10 µL.

The LC was coupled to a triple-quadrupole mass spectrometer Finnigan TSQ Quantum Discovery (Thermo Electron Corp.). The ion source was operated in the electrospray ionization (ESI) positive mode resulting in protonated molecular ions (as for type B trichothecenes) or sodium adduct ions (as for type A trichothecenes). Most trichothecenes were quantified by tandem MS in the selected reaction monitoring (SRM) mode using characteristic transitions from the parent ion. The only exceptions were DAS and MAS, which did not show reproducible fragmentation and, therefore, were determined by single stage MS in the single ion monitoring mode.

Cereal based food samples were purchased from local markets, finely ground, and thoroughly homogenized. About 3–5 grams of homogenous material was weighed in an Erlenmeyer flask and labelled standards were added. Subsequently, trichothecenes were extracted by a mixture of water-acetonitrile (16:84, v/v, 15 mL) followed by filtration and clean-up over MultiSep® 225 Trich cleanup columns (Romer Labs Inc., Union, USA). The purified extracts were evaporated at 60 °C *in vacuo*, redissolved in mobile phase for LC-MS analysis (100 µL), membrane filtered, and subjected to LC-MS/MS (*9*).

For matrix-assisted external calibration, homogenous samples of oat flakes devoid of mycotoxins were spiked with solutions of DON and T-2 toxin at different concentration levels and analyzed in triplicate as described above. The same solutions of DON and T-2 toxin were used for direct external calibration after dilution with water. To determine analyte losses during sample preparation labelled internal standards were added to a series of samples at different stages of analysis, i.e., after filtration, after column clean-up, and after evaporation. Analysis of each stage was performed in triplicate.

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Results and Discussion

To date, [$^{13}C_{15}$]-DON is the only trichothecene mycotoxin that is commercially available as a labelled internal standard. To develop SIDAs for all other relevant trichothecenes (Figure 1) we synthesized these compounds as isotopologues using carbon-13 labelling of the acetyl moieties.

The four labelled type A trichothecenes and [$^{13}C_2$]-4-AcNIV were easily obtained by alkaline hydrolysis of the peracetylated compounds. The preferred formation of the desired mono- and di-acetates out of a number of potential isomeric compounds is due to the fact that they are more stable under alkaline conditions than the respective isomers. In a like manner, alkaline hydrolysis of [$^{13}C_4$]-DON-3,15-diacetate resulted in only one monoacetate, namely the more stable [$^{13}C_2$]-3-AcDON isomer. In contrast to this, direct [^{13}C]-acetylation of DON by using sub-stoichometric amounts of [$^{13}C_4$]-acetic anhydride yielded mainly [$^{13}C_2$]-15-AcDON and [$^{13}C_4$]-DON-3,15-diacetate along with unreacted DON, but only traces of [$^{13}C_2$]-3-AcDON (Figure 2A). Therefore, synthesis of both isomers was only possible after separating a reaction mixture containing [$^{13}C_2$]-15-AcDON and [$^{13}C_4$]-DON-3,15-diacetate using preparative LC and subsequent alkaline hydrolysis of the diacetate (Figure 2B).

Using the seven synthesized isotopologic trichothecenes and [$^{13}C_{15}$]-DON, stable isotope dilution assays were developed applying LC-MS/MS. Appropriate LC conditions allowed the separation of all trichothecenes under study within one run (Figure 3). Chromatographic separation was essential to distinguish

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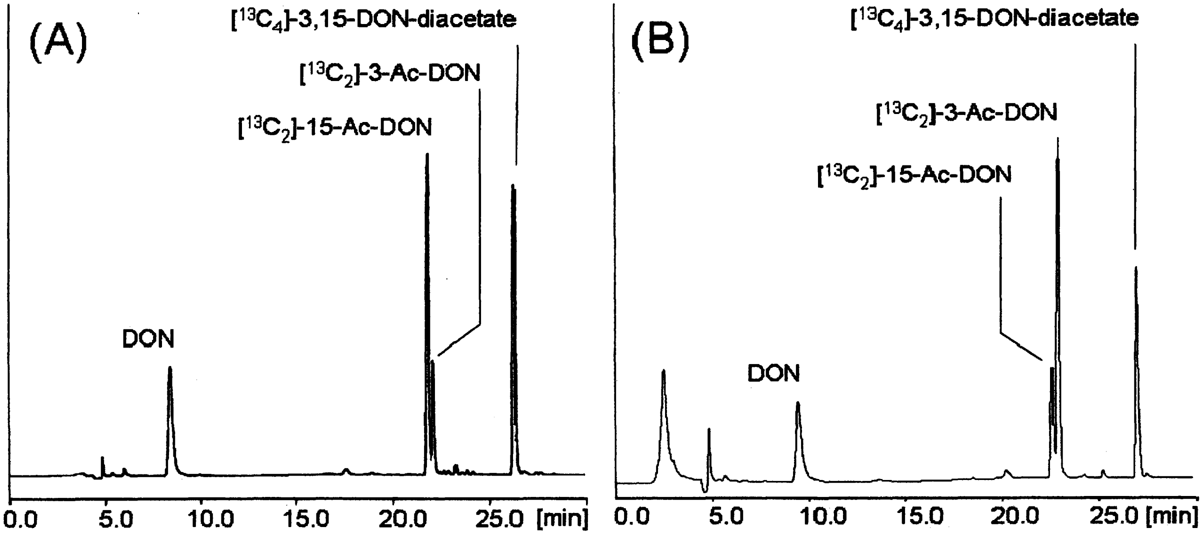

Figure 2. (A) Reaction of [$^{13}C_4$]-acetic anhydride with an excess of DON; (B) alkaline hydrolysis of [$^{13}C_4$]-DON-3,15-diacetate.

between the acetyl-DON isomers showing the same signal of the protonated molecule and the same fragmentation during MS/MS.

Positive electrospray ionisation transformed the type B trichothecenes into protonated ions and the type A trichothecenes into sodium adducts. Except for MAS and DAS all substances showed reproducible fragmentation of the parent ions in MS/MS experiments. MAS and DAS did not fragment and, therefore, had to be quantified with single stage MS.

Validation of the developed method resulted in recoveries of 90 to 127 % depending on the toxin under study. The coefficient of variation in inter-assay precision studies (n = 3) ranged between 2.6 and 8.0 %, also differing between the single trichothecenes. Limits of detection (LOD) and limits of quantitation (LOQ) were determined by the method suggested by Hädrich and Vogelgesang (*10*), which considers also precision, matrix effects, and recovery of additions near the LOD. In this way, LODs spanned a range between 1 µg/kg (DAS) and 30 µg/kg (MAS) and LOQs were found to range between 4 µg/kg (DAS) and 80 µg/kg (MAS).

The advantages of SIDA became obvious when comparing the analytical results of a naturally contaminated sample (oat flakes) (a) using SIDA, (b) using external calibration, and (c) using external matrix-assisted calibration (Figure 4). External calibration resulted in about 50 % of the concentration of DON and about 30 % of the concentration of T-2 toxin compared to the values obtained using SIDA. This effect could be attributed either solely to incomplete recovery or to a combination with matrix dependent ion-suppressing effects in the ESI interface during LC-MS/MS analysis. To study these effects, labelled standards were added to an extract of oat flakes only after clean-up, which enabled calculation of the loss of analyte during sample preparation, whereas the labelled standards compensated for the ion-suppression in MS/MS analysis. The results of this experiment showed that during sample preparation the loss of DON was about 60 %, in contrast to T-2 toxin, which was recovered almost completely. Further studies (data not shown) revealed that this effect was not a consequence of the clean-up process itself in the first place, but rather of different extraction behaviours from the matrix with acetonitrile-water due to differing polarity of the analytes. From these results it could be concluded that at least in the case of oat flakes a remarkable ion-suppression did not occur for DON. However, the latter effect might be responsible for the low result of T-2 toxin in external calibration as it appears to be almost completely extracted from the matrix.

Using external matrix-assisted calibration the content of DON correlated with the SIDA value indeed, but standard derivatization was poor in triplicate analysis. Furthermore the concentration of T-2 toxin determined by external matrix-assisted calibration was 25 % higher than the value obtained using SIDA, indicating signal-interference with matrix compounds during LC-MS/MS analysis.

Taken together, these results clearly indicate that the use of stable isotope labelled standards is the method of choice to overcome analytical difficulties,

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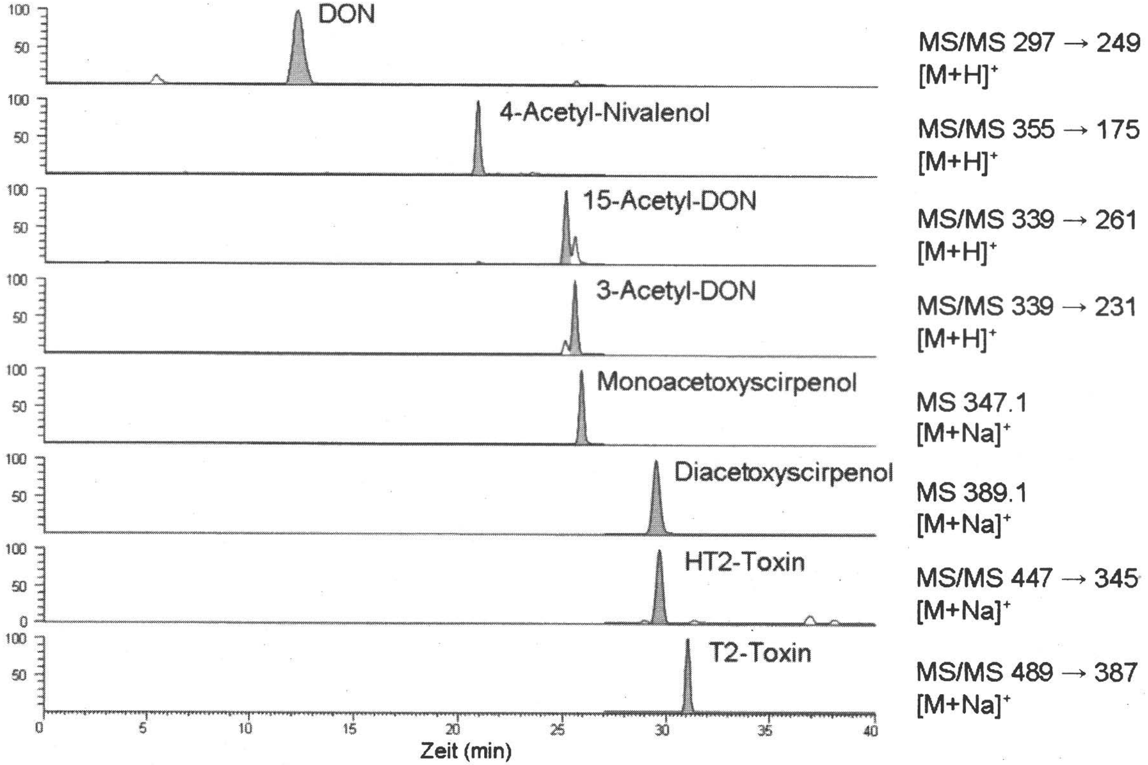

Figure 3. LC-MS run of eight trichothecenes (only traces of unlabelled comp. shown).

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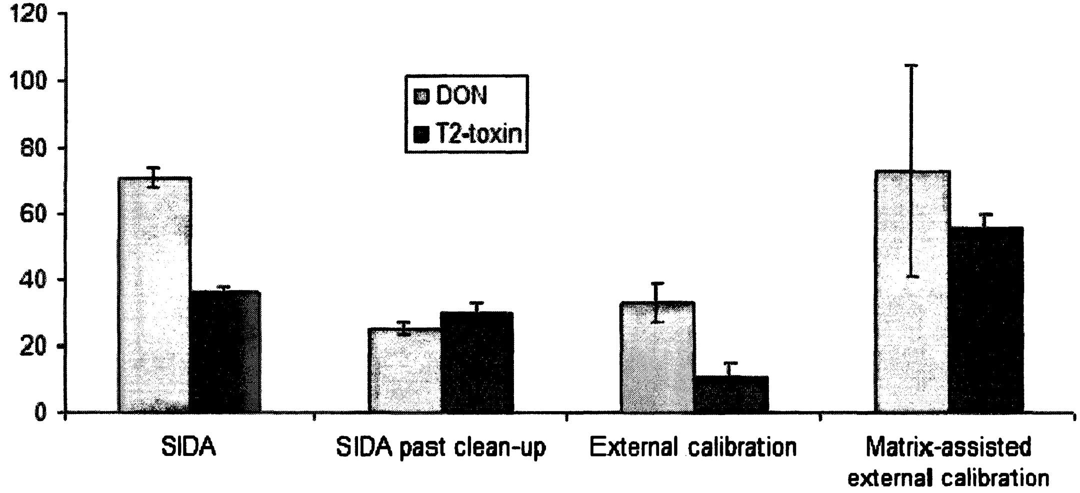

Figure 4. Difference between analytical methods and effects of clean-up on the concentration of DON and T-2 toxin.

time consuming method optimization, and varying matrix effects to achieve accurate analytical results for trichothecene contamination of food.

As an application of the developed SIDA a series of food samples obtained from local markets were analyzed for trichothecene mycotoxins. Regarding type A trichothecenes, we found high contamination of oat and oat products with T-2 toxin and HT-2 toxin especially, whereas MAS was not detectable and DAS was present only in traces (Table I). In oat flakes the sum of T-2 toxin and HT-2 toxin exceeded 50 μg/kg in most cases. DAS could be found in different agricultural commodities such as maize, spelt, and potatoes, but its concentrations were lower than 10 μg/kg in general.

Type B trichothecenes occurred more frequent and generally in higher concentrations than type A trichothecenes. None of the analyzed wheat or maize samples was devoid of DON, which is in fact alarming, although the absolute levels did not exceed the legal limit for DON in the European Union. In maize, the concentration of 15-AcDON was sometimes higher than that of DON, pointing to the need for methods that can detect more than one mycotoxin simultaneously.

Of special interest are the concentrations of type B trichothecenes in processed foods. In breakfast cereals DON was regularly found in amounts up to 280 μg/kg, but also 3-AcDON was detectable in 3 out of 11 samples. Whereas concentrations of DON were relatively low in bread, some samples of maize chips, cookies, and pasta were much more highly contaminated with DON and also with 3-AcDON and 15-AcDON (Table II).

A bottom fermented (lager) beer made from barley contained DON only at levels of 8 μg/kg, whereas in a top fermented beer made from wheat, DON could be quantified in concentrations up to 20 μg/kg. Although the contamination level appears rather low, the total DON intake can be significant when considering the higher consumption of beer compared to other food such as maize chips or cookies.

The bottom line is that contamination of cereal products with trichothecenes is still high. Often there is more than one trichothecene mycotoxin present in the food sample. For the protection of consumers it is therefore important to apply sensitive and reliable analytical methods that are able to detect different trichothecenes in varying food matrices simultaneously, together with a reasonable analytical effort. As we have demonstrated, SIDAs are very suitable to meet all the demands mentioned.

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Table I. Concentrations of Trichothecenes in Different Cereal Products *)

Type A	*No. of Samples*	*No. of Positive Samples (concentration in µg/kg)*		
Sample		*T-2 toxin*	*HT-2 toxin*	*DAS*
Oat flakes	10	6 (4 – 50)	5 (40 – 100)	2 (~1)
Oat kernels	1	1 (~3)	0	0
Oat cookies	2	0	0	2 (~1)
Maize flour	1	1 (~3)	0	1 (~2)
Maize grit	1	0	0	1 (5)
Spelt kernels	1	0	0	1 (10)
Potatoes	1	0	0	1 (4)

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Type B	*No. of*	*No. of Positive Samples (Concentration in µg/kg)*		
Sample	*Samples*	*DON*	*3-AcDON*	*15-AcDON*
Wheat, grown				
(a) conventionally	12	12 (35 – 300)	1 (~7)	1 (~4)
(b) organically	2	2 (6 – 30)	1 (~6)	0
Maize, grown				
(a) conventionally	4	4 (8 – 160)	1 (~4)	3 (30 – 50)
(b) organically	2	2 (6 – 30)	0.	2 (10 – 20)
Barley kernels	1	1 (35)	0	0
Oat kernels	2	0	0	0
Rye flour	3	3 (20 – 50)	0	0
Spelt kernels	1	1 (40)	0	0

0 = concentration below limit of detection; ~ = concentration below limit of quantification.

*) MAS and 4-AcNIV were not present in any analyzed sample.

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Table II. Concentrations of Type B Trichothecenes in Processed Food

Sample	*No. of Samples*	*No. of Positive Samples (Concentration in μg/kg)*		
		DON	*3-AcDON*	*15-AcDON*
Breakfast cereals	11	11 (15 – 280)	3 (10 – 25)	0
Toasted bread	1	1 (45)	0	0
Whole wheat bread	1	1 (40)	0	0
Maize chips	3	3 (30 - 320)	1 (~ 2)	1 (35)
Cookies	2	2 (15 – 80)	1 (20)	0
Pasta	3	3 (30 – 140)	1 (~ 2)	2 (20 – 30)
Beer (bottom fermented)	1	1 (8)	0	0
Wheat beer	4	4 (7 – 20)	0	0
Baby formula	1	1 (15)	0	0

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Chapter 14

Determination of Fumonisin B_1 in Botanical Roots

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Fumonisins are toxins produced mainly by the molds *Fusarium verticillioides*, *F. proliferatum*, and several other *Fusarium* species that grow on agricultural commodities in the field or during storage. More than ten types of fumonisins have been isolated and characterized. Fumonisin B_1 (FB_1), B_2 (FB_2), and B_3 (FB_3) are the major fumonisins produced. FB_1 is the most prevalent and most toxic. Recently, fumonisins have been found in botanical roots. Fumonisins have produced liver damage and changes in the levels of certain classes of lipids, especially sphingolipids, in all animals studied.

Many analytical methods for determining fumonisins in foods have been published. Among the most common of these methods are liquid chromatographic (LC) separation with fluorescence detection, enzyme-linked immunosorbent assay (ELISA) and LC-mass spectrometry (MS). In our laboratory, the botanical roots of ginseng, ginger, turmeric and kava-kava were extracted with a mixture of methanol and water, followed by cleanup on an immunoaffinity column (IAC). FB_1 was then derivatized, separated, and determined by LC with fluorescence detection. Recoveries of FB_1 added to ginseng, ginger, turmeric, and kava-kava roots at levels ranging from 0.05 to 2 μg/g, were >75% except for turmeric at a spiked level of 0.05 μg/g. ELISA was also applied to screen these roots for FB_1.

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ACS Symposium Series; American Chemical Society: Washington, DC, 2008.

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Numerous dietary supplements are commercially available worldwide to promote health, increase longevity, or lose weight (*1*). Dietary supplement sales in the U.S. were approximately twenty billion dollars in 2005 (*2*). Some botanical supplements such as garlic, ginger, and turmeric have been used as food and condiments for centuries. Botanicals are generally regarded as safe for consumption, however, they might contain chemical contaminants such as heavy metals, pesticides, and mycotoxins. Fumonisins have been found in medicinal wild plants in South Africa (*3*) and also in herbal tea and medicinal plants in Turkey (*4*).

Fumonisins are a group of structurally related compounds produced by several species of *Fusarium*; *Fusarium verticilliodes*, *F. proliferatum* and *F. nygami* are the main fumonisin producing strains (*5*). This group of mycotoxins is characterized by a 19-20 carbon aminopolyhydroxy-alkyl chain which is diesterified with two tricarballylic acid groups. Since 1988 about 28 fumonisin analogs have been characterized and classified into four main groups: A, B, C, and P series (*6*). The fumonisin B analogs (FBs), comprising toxicologically important FB_1, FB_2, and FB_3, are the most abundant naturally occurring fumonisins, with FB_1 usually being found at the highest levels in naturally contaminated grains (*7*).

FBs have been associated with health problems in animals such as cancer in rodents (*8*), equine leukoencephalomalacia (ELEM) in horses (*9*), and pulmonary edema in swine (*10*). Epidemiology studies have correlated human consumption of fumonisin contaminated corn and esophageal cancer (*11*). For these reasons the International Agency for Research on Cancer (IARC) has classified FBs as possible carcinogens (*12*). In 2001, the US Food and Drug Administration (FDA) issued a guidance document to industry recommending a maximum allowable level of FBs in corn used in the production of human foods and animal feeds (*13*). The non-mandatory guidelines range from 2 μg/g to 4 μg/g depending on the corn product.

The most commonly used analytical method for determining FBs in a wide variety of matrices is LC with precolumn fluorescence derivatization of fumonisins with *o*-phthaldialdehyde (OPA)/mercaptoethanol (ME). ELISA methods based on monoclonal and polyclonal antibodies specific for fumonisins are being used as a cost effective high throughput screening technique. LC-MS methods have been used for quantitation as well as for validation of results obtained using LC or ELISA methods. The objectives of this study are to determine FB_1 in botanical roots using all three techniques.

Materials and Methods

Finely ground plant materials for the recovery study - ginseng (*Panax quinquefolius*), kava-kava, and turmeric - were purchased from Schumacher

Ginseng (Marathon, WI). Finely ground ginger (*Zingiber officinale*) for the recovery study was purchased from McCormick (Baltimore, MD). FB_1 with purity >95% (based on data obtained with LC, nuclear magnetic resonance (NMR),and LC-MS analyses) was isolated from fungal cultures and purifed in our laboratory. All solvents were suitable for LC analysis and were purchased from Baker Chemicals. Glacial acetic acid and sodium bicarbonate were obtained from Fisher Scientific; phosphate buffered saline (PBS) 10 mM, OPA, ME, and Tween 20 were from Sigma-Aldridge (St. Louis, MO).

Orbital shaker (VWR DS-500E, VWR International, Bridgeport, NJ); centrifuge (Allegra X – 22R, VWR International, Bridgeport, NJ); glass microfiber filter paper, 11 cm (Whatman 934AH, Whatman Inc., Clifton, NJ); and immunoaffinity columns (G1008, wide bore Fumonitest columns, Vicam Corp., Watertown, MA) were used. The LC system consisted of a Waters 600 E pump, Waters 717 plus injector, Waters 2475 fluorescence detector (set at excitation 335 nm and emission 440 nm), Empower 2 control and data system (Waters Corp., Milford, MA), and a C18 IP LC column (Beckman 235335, Utrasphere, 4.6 x 250 mm, 5 µm, Beckman Instruments, Inc., Fullerton, CA). The limit volume inserts were from Waters Corp. ELISA kits - Veratox for fumonisins HS - were from Neogen Corp. (Lansing, MI). The LC-MS system consisted of an Agilent 1100 series LC (Agilent Technologies, Santa Clara, CA) connected to an API 5000 triple-quadrupole mass spectrometer via a turbospray ion source (Applied Biosystems, Framingham, MA) and a Waters YMC L-80 C-18 LC column, 2 x 250 mm, 4 µm particle size.

Extractions: A 5 g test portion from each plant material was extracted using 25 mL methanol-acetonitrile-water (25:25:50, v/v/v) with shaking for 10 min at 400 rpm. The mixture was centrifuged and 7 mL of the supernatant was mixed with 28 mL 10 mM PBS containing 1% Tween 20. The mixture was filtered through microfiber paper and 25 mL of filtrate was collected in a 25 mL graduated cylinder.

Immunoaffinity column (IAC) chromatography: The column was preconditioned with 5 mL PBS followed by the addition of the 25 mL filtrate. The column was then washed with 10 mL PBS. FB_1 was eluted with two 1.0 mL portions of methanol-water (8:2, v/v). The eluate was collected into a 4 mL vial and evaporated to dryness in a SpeedVac evaporator.

Liquid chromatography (LC): The dried eluate was dissolved in 200 µL acetonitrile-water (1:1, v/v). The stock standard solution was prepared by dissolving 10 mg FB_1 in acetonitrile-water (1:1, v/v). The working standard solutions were prepared by diluting the stock solution to 2, 1, 0.5, 0.25, 0.125 and 0.625 µg/mL with the same solvent. OPA reagent was prepared by dissolving 50 mg OPA in 1 mL methanol then adding 49 mL 0.06 M sodium tetraborate and mixing. Before use, 20 µL ME was added to 5 mL of OPA reagent and mixed. The LC column was equilibrated with a mobile phase of acetonitrile-water-acetic acid (950:1050:20, v/v) at a flow rate of 1.0 mL/min.

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The autosampler was programmed to transfer 70 µL derivatization reagent into a 400 µL limited volume insert containing 30 µL test sample extract; 100 µL of the mixture was drawn into the syringe at 5 µL/s; the mixture was dispensed back into the sample vial. The drawing and dispensing were performed 3 times; then 30 µL of the mixture was injected onto the LC column. The retention time for FB_1 was about 12 min. Calculation of FB_1 concentration in test samples was based on peak areas compared with those of the standards.

ELISA assay: All reagents were included in the commercial kit. The assay procedure was provided by the manufacturer and is briefly described below. The test sample was extracted with 70% methanol and the mixture was shaken and centrifuged. A portion of the supernatant was diluted with water. The diluted extract was mixed with an equal volume of antibody-enzyme conjugate solution and then added to the wells coated with antibodies. After incubating at room temperature the wells were emptied, washed with water, additional substrate added, incubated again, stopping reagent added and then the results were read with an ELISA reader.

LC-MS/MS analysis: Eluates from the immunoaffinity column were not driedfor this analysis but were transferred to autosampler vials and injected directly into the LC-MS/MS system. The mobile phase used was the same as for the LC method above. The flow rate was 200 µL/min and the column heater was set at 30 °C. The mass spectrometer was operated in positive electrospray ionization (ESI^+) mode and ions indicative of FB_1 were observed using multiple reaction monitoring (MRM). High purity nitrogen was used for the curtain gas, ESI nebulizing gas and the collision gas for collision activated dissociation (CAD). The ion spray voltage was set at 5 kV and the source block and desolvation temperatures were 140 and 450 °C, respectively. The two fragment ions of reactions monitored resulted from the CAD of the protonated molecular ion at *m/z* 722 to *m/z* 352 and to *m/z* 334 respectively. Other instrumental conditions and settings included: CAD gas 6; Curtain gas 15; nebulizing gas 30; desolvation gas 30; declustering potential (DP) 110 V; exit potential (EP) 10 V; collision energy (CE) 40 V for *m/z* 722 > 352 and 30 V for *m/z* 722 > 334.

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Results and Discussion

LC chromatograms of added FB_1 in ginger roots and FB_1 standards are shown in Figure 1. Chromatograms of the other roots were similar to those of the ginger roots. The OPA reagent reacts with all primary amines present in the sample extract and can lead to interferences that are difficult to separate by LC. Often modification of mobile phase is necessary. In general, the reduction of the acetonitrile concentrations to about 46% resolves some of the problem. The derivatization reagent composition and extract to reagent ratio were modified from the AOAC International Official Method (*14*). The concentrations of OPA,

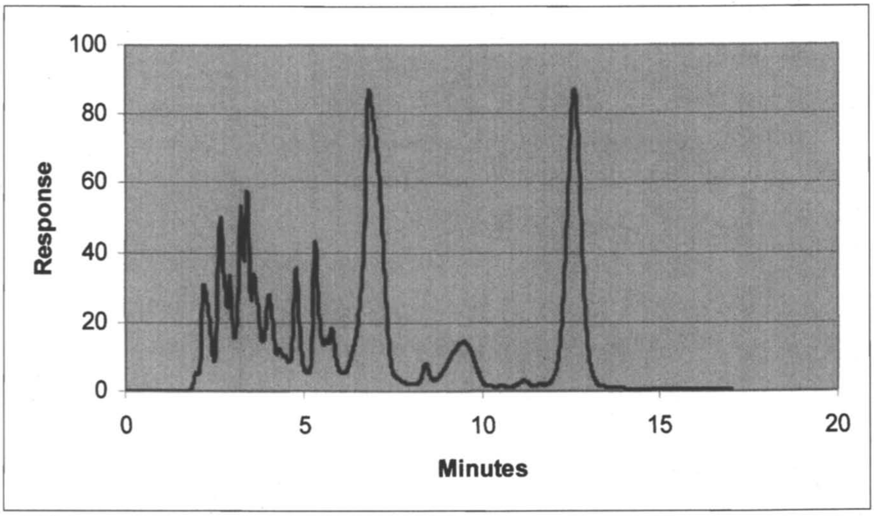

Figure 1. Chromatogram of added fumonisin B_1 in ginger roots at 0.1 μg/g.

sodium tetraborate and ME in the derivatization reagent were much lower and the extract to reagent ratio was changed from 1:5 to 1:2. The LC chromatogram of reagent blank showed much less response for reagent peaks at retention times near the FB_1 peak area than the AOAC International Official Method. ME must be added to the OPA reagent daily before use due to its volatility and the small volume of ME in the derivatization reagent.

Ginseng, ginger, kava-kava, and turmeric found to contain <0.01 μg/g of FB_1 were used for recovery studies. The limit of detection of the method was about 0.01 μg/g (standard at signal to noise ratio, 5:1). Results for recovery studies of FB_1 in botanical roots using IAC/LC are summarized in Table I. Average recoveries of FB_1 added to all roots were >75% except kava-kava at an added level of 0.05 μg/g. The lowest level in our recovery study was 0.05 μg/g and this is considered as the limit of determination.

The use of OPA as derivatization reagent has some drawbacks: the mixing of test extract before injection onto the LC column and the stability of the derivatives. A dead volume of extract in the sample vial is required because the openings of the needles of the injectors are on the side instead of at the end. Two different types of LC autoinjectors were investigated using FB_1 standards. The first injector mixed the derivatizing reagent and test solution by repetitive drawing and dispensing the entire amount of extract and reagent mixture in and out of the syringe before injection. The type 1 autoinjector withdrew reagent, then test solution and reagent again into the injector loop; mixing was by diffusion with delayed injection. For the type 1 autoinjector various extract and

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Table I. Recoveries of Fumonisin B_1 (µg/g) Added to Botanical Roots by the immunoaffinity Column/Liquid Chromatography Method

Roots[1]	*Added*	*Recovery*[2] *%*	*SD*[3]	*RSDr*[4]
Ginseng	0.05			
	0.10			
	0.25	89	13	14
	0.50	90	5	5
	1.00	94	8	9
	2.00	98	3	3
Ginger	0.05	84	21	25
	0.10	110	9	8
	0.25	110	9	8
	0.50	96	6	6
	1.00	75	5	6
	2.00	89	2	3
Turmeric	0.05	92	8	9
	0.10	101	4	4
	0.25	98	5	5
	0.50	110	8	7
	1.00	96	15	15
	2.00	85	1	1
Kava-kava	0.05	60	26	43
	0.10	77	25	33
	0.25	110	26	24
	0.50	111	8	7
	1.00	95	15	15
	2.00	84	6	7

[1] All roots were found to contain <0.01 µg/g.

[2] Four replicate analyses for each spiking level.

[3] Standard deviation.

[4] Relative standard deviation.

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Table II. Recoveries of Fumonisin B_1 (µg/g) Added to Botanical Roots by the Enzyme-linked Immunosorbent Method

Roots	*N*[1]	*Added*	*Found*	*Recovery, %*[2]	*SD*[2], *%*	*RSDr*[3], *%*
Ginseng	15	0	40			
	12	0.10	122	122	38	31
	18	0.20	145	73	21	29
	22	0.40	325	81	19	24
	24	0.60	428	71	11	15
Ginger	8	0	19			
	6	0.10	135	135	13	10
	12	0.20	198	99	11	11
	12	0.40	373	93	25	27
	17	0.60	616	103	19	18
Kava-	6	0	58			
kava	6	0.10	240	240	13	6
	6	0.20	235	117	4	4
	9	0.40	444	111	13	13
	8	0.60	558	93	6	6
Turmeric	4	0	193			
	6	0.10	357	164[4]	14	14
	6	0.20	513	160[4]	7	7
	6	0.40	686	123[4]	8	8
	8	0.60	725	89[4]	10	10

[1] Number of analyses.

[2] Standard deviation.

[3] Relative standard deviation.

[4] Recoveries of FB_1added to turmeric were corrected for the 0.193 µg/g in control; no corrections were made for the recoveries of added FB_1 in the other roots.

derivatization ratios, various withdrawing and dispensing times, and various injection volumes were investigated. It was found that the optimum conditions for derivatization were mixing 3 parts of test solution and 7 parts of reagent, repeating withdrawal and dispensing 3 times, and injecting 35 μL of the mixture into the LC column. The total mixing time was about 9 min. For the second type autoinjector, various reagent-test solution-reagent drawing into the loop and delay times were studied. But optimum and reproducible results were obtained. The type 1 autoinjector with mixing capability gave reproducible results, while, inconsistent results were obtained using the type 2 autoinjector. Therefore type 1 autoinjector was use to develop this method. The use of the limited volume inserts instead of the 1 mL vials is essential. This avoids mixing large volume of extract and derivatization reagent for the mixture to reach the height of the injector opening. The mixing time is proportional to the volume of the mixture. The fluorescence intensity of the OPA derivative is time dependent. The fluorescence intensity decreases about 15 % after 10 min (*15*).

Of several batches of powdered ginseng, ginger, kava-kava, and turrmeric analysed, two turmeric samples were found to contain FB_1 at levels of 0.10 and 0.15 μg/g. LC-MS/MS was used to confirm the identity of FB_1 in extracts of two turmeric samples. Confirmation was based on comparison of retention time and multiple reaction monitoring (MRM) data with those of standards.

One ELISA method was evaluated. Ginseng, kava-kava, and ginger that were found to contain <0.1 μg/g of FB_1 by the LC method were used. Turmeric was found to contain 0.193 μg/g of FB_1 by ELISA while the LC method found the same test sample to contain FB_1 at 0.10 μg/g. Results for the recovery study are shown in Table II. Recoveries of FB_1 added to botanical roots at 0.1 – 0.6 μg/g were 71–240%. Our results indicate ELISA methods are prone to overestimate FB_1 at levels <0.2 μg/g. Likely reasons may be that the antibodies can cross-react with other fumonisins, or cross-react with other compounds in the matrix. From this study ELISA appeared to be suitable for FB_1 determination at levels >0.6 μg/g.

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Summary

The wide bore IAC packed with anti-FBs is suitable for FB_1 determination in ginseng, ginger, kava-kava and turmeric at level >0.05 μg/g. The ELISA method shows non-specific binding and gave results for FB_1 greater than the reagent blanks (>0 μg/g but <0.05 μg/g) for all the roots examined, but it is suitable for these roots at FB_1 >0.10 μg/g.

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Chapter 15

Development of an Immunoaffinity Column for the Determination of T-2 and HT-2 Toxins in Cereals Using Liquid Chromatography with Fluorescence Detection

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With new European Union (EU) mycotoxin regulations under consideration for T-2 toxin (T-2) and HT-2 toxin (HT-2) in cereals, there is a need for new analytical methods to detect and quantify these toxins accurately. It is often difficult to quantify T-2 and HT-2 in cereals due to interfering components and pigments in the samples, which can often mask T-2 and HT-2 peaks in a liquid chromatography (LC) chromatogram leading to poor recoveries and sensitivity. An improved method has been developed using an immunoaffinity column in conjunction with LC-fluorescence. The protocol involved extraction with 90% methanol and filtration prior to dilution of the sample with water (for maize, wheat, and barley) or with 2% sodium chloride (for more complex oat samples) and application onto the immunoaffinity column. Use of the 2% sodium chloride for dilution of the oats extract was developed in conjunction with Nestlė Research Centre in Switzerland and facilitated precipitation of proteins from the sample and removal of interfering components, resulting in improved chromatography for this matrix. This method was shown to be rapid and easy to use with a limit of detection of 1 ppb for HT-2 and 10 ppb for T-2 in cereals, which is significantly lower than proposed EU legislative limits (*1*). Furthermore the sensitivity of the method can be improved by passing a larger volume of sample extract through the immunoaffinity column prior to analysis by LC. Excellent recoveries of 70-105% for T-2 and between 100-119% for HT-2 were obtained with wheat, barley, maize, and oats and the method was found to be robust and sensitive. It can therefore be concluded that the method using immunoaffinity columns will meet forthcoming EU legislative limits and will satisfy recommended method performance criteria for these toxins.

Currently no official method exists for the determination of T-2 toxin (T-2) and HT-2 toxin (HT-2); before legislation can be introduced it has been recommended that analytical methods for T-2 and HT-2 are required with acceptable recovery, accuracy, and analytical precision. There is also thought to be a need for reference materials, which can be used to check consistency, reliability, and traceability of analytical methods (*2*). These materials and methods can then be used in international collaborative studies for T-2 and HT-2 to ensure that proposed legislative levels can be met. EASI-EXTRACT® T-2 and HT-2 immunoaffinity columns were developed to provide a suitable method for detection of T-2 and HT-2 in cereals. The columns were assessed in conjunction with a liquid chromatography (LC)-fluorescence system in order to improve the detection of T-2 and HT-2 in cereals by providing cleaner chromatography and better sensitivity than the more traditional solid phase extraction columns.

T-2 and HT-2 occur in a wide range of cereal products including wheat, oats, and barley and often co-occur with other trichothecenes (*3*). These mycotoxins are often found in cereals and feed and can cause acute symptoms in livestock and poultry, including vomiting, feed refusal, and poor weight gain. They can also have chronic effects on animals such as immunosupression, oral lesions, and lethargy (*4*). T-2 and HT-2 are type A trichothecenes which means that LC with UV detection is not generally applicable since they lack a keto group at the C-8 position (*5*), although a variety of post-column derivatisation methods have been developed, including those involving detection of *p*-nitrobenzoate or diphenylindenone sulfonyl esters of T-2 (*6*). For this reason, GC analysis is generally the chosen method for the determination of T-2 and HT-2. Most methods for type A trichothecenes are based on trimethylsilylation or fluoroacylation for derivatization to increase volatility and sensitivity. Detection is then carried out using mass spectrometry (MS) (*7*) or with an electron capture detector (ECD) for the fluoroacylated trichothecenes. The formation of fluoroacyl derivatives by trifluoroacetic anhydride (TFA), pentafluoropropionyl (PFP), or heptafluorobutyryl (HFB) derivatization increase the sensitivity of the LC-ECD procedure (*6*). Other newer methods for detection of trichothecenes by LC-MS/MS have also been developed using solid phase extraction columns or multifunctional cleanup columns (5, *8*).

For extraction of T-2 and HT-2 from cereal samples different extraction solvents can be employed, including acetonitrile-water or methanol-water, and extraction can be facilitated by shaking or blending. Sample clean up is then often carried out using silica gel, florisil, cyano and C_{18} solid phase extraction columns. Typical detection limits for quantitative determination of T-2 and HT-2 in cereals are 3 ng/g (T-2, LC-MS), 1 ng/g (HT-2, LC-MS), 10 ng/g (T-2 and HT-2, GC-MS) and 10 to 50 ng/g (T-2 and HT-2, GC-ECD). Typical recoveries can range from 70-120% (*6*). The problem with these techniques, however, is that the associated equipment is very expensive and is not widely available for use by smaller food companies and analytical laboratories. In addition highly experienced analysts must be employed to use and maintain these systems.

Furthermore some complex food matrices such as animal feed and oats can cause quenching of the T-2 and HT-2 signals making accurate quantification difficult. Analysis by LC is more widely available to smaller companies and many laboratories already use immunoaffinity columns in conjunction with LC for analysis of other mycotoxins. Moreover, 1-anthroylnitrile (1-AN) has been shown to be an efficient labelling reagent for the determination of T-2 by LC-fluorescence (*9*). The use of a highly selective immunoaffinity column for T-2 and HT-2 in conjunction with LC-fluorescence will make this type of analysis accessible to a greater number of laboratories.

The EASI-EXTRACT® T-2 and HT-2 immunoaffinity columns use a monoclonal antibody bound to a gel support, which is highly specific for both T-2 and HT-2. The column not only cleans up sample extracts prior to quantification by LC-fluorescence but also concentrates the toxin, therefore improving detection and sensitivity. As with previous methods, T-2 and HT-2 were extracted from cereals using 90% aqueous methanol (v/v) before filtering and dilution with deionised water followed by immunoaffinity column cleanup. The toxin was released from the immunoaffinity column with methanol and the eluate evaporated to dryness before derivatization with 1-anthroylnitrile in the presence of 4-dimethylaminopyridine for fluorescence detection (*9*).

Materials and Methods

Reagents and Consumables

4-Dimethylaminopyridine (DMAP) (Sigma, product code: D5640-25G); 1-anthroylnitrile (1-AN) (WAKO, product code: 017-12101); toluene (high grade, Fluka, product code: 89676); filter paper (Whatman N° 113 or N° 4); glass microfiber filter paper (Sartorius MGB); T-2 standard (Sigma, product code: T4887); HT-2 standard (Sigma, product code: T4138); and T-2 and HT-2 immunoaffinity columns (EASI-EXTRACT®, R-Biopharm Rhône Ltd., product code: P43 and P43B).

Sample Preparation

Test portions of 50 g cereal samples were extracted with 250 mL of methanol- water (9:1, v/v) plus 5 g of sodium chloride by blending at high speed for 2 min then filtering through a Whatman No. 113 filter paper.

Immunoaffinity Column Clean up and Derivatization Procedure

A 7 mL aliquot of filtrate was diluted with 28 mL of deionised water for wheat, barley, and maize samples. For more pigmented samples like oats, 7 ml of filtrate was diluted with 28 mL of 2% sodium chloride solution; the solution was gently shaken and left to settle for 5 min for precipitation of proteins from the extract to occur. The diluted filtrates were then further filtered through a Sartorius MGB glass microfibre filter paper. Twenty five mL of solution was passed through the EASI-EXTRACT® T-2 and HT-2 immunoaffinity columns at a slow flow rate of 3 mL per min. The immunoaffinity column was then washed with 20 mL of deionised water to remove any unbound residues from the column. Toxin was released from the immunoaffinity column using 1.5 mL methanol and the eluate was evaporated to dryness at 50 °C under a gentle stream of air. Fifty μL of 1-anthroylnitrile and 50 μL of 4-dimethylaminopyridine were added and mixed gently by vortex for 1 min; the mixture was placed on a heating block at 50 °C for 15 min. cooled in an iced water bath for 15 min, and evaporated to dryness at 50 °C under a gentle stream of air. The residue was reconstituted in 1 mL of mobile phase and mixed on a vortex mixer for 30 sec, before injecting 100 μL into the LC system.

In-House Validation

The method using EASI-EXTRACT® immunoaffinity columns with LC-fluorescence detection was assessed using a range of spiked cereal extracts including extracts of maize, barley, wheat, and oats spiked at 100 ppb (total T-2 and HT-2, 1:1) to check the performance of the immunoaffinity columns and the quality of the LC traces following column clean-up (Table II). The performance of the complete method, including extraction with methanol-water (9:1, v/v) and

Table I. LC Conditions

Guard cartridge:	Supelco guard filter (0.5 μm).
Analytical column:	Analytical column, Luna 5μ Phenyl-Hexyl LC column (150 mm x 4.6mm, 5μm).
Mobile phase: a binary gradient was applied	Initial composition = acetonitrile-water (70:30, v/v) 5 min = acetonitrile- water (70:30, v/v) 15 min = acetonitrile- water (85:15, v/v) 25 min = acetonitrile- water (85:15, v/v) 27 min = 100% acetonitrile 32 min = 100% acetonitrile 35 min = acetonitrile- water (70:30, v/v)
LC pump	To deliver mobile phase at flow rate:1.0 mL/min
Fluorescence detector:	Excitation: 381nm; emission: 470 nm
Column heater:	Maintain guard and analytical columns at 40 °C
Integrator	From preferred supplier
Injector	Autosampler /Rheodyne valve
Injection volume:	100 μL

immunoaffinity column cleanup, was assessed on a range of spiked cereal samples. Samples of wheat, maize, and barley were spiked directly with 100 ppb total T-2 and HT-2 (1:1) (Table III). A naturally contaminated barley reference material was also analysed using the new method (Table IV) and results compared to GC-MS combined with trimethylsilyl derivatization (*7*); the same sample was then analysed over several days using LC-fluorescence.

Table II. Extracts Spiked with 100 ppb total toxin (T-2 and HT-2, 1:1)

Sample	*No. of Analyses*	*Average ppb*		*Average Recovery, %*		*% CV*	
		T-2	HT-2	T-2	HT-2	T-2	HT-2
Pearl barley	(n=3)	51	51	102	102	10.0	10.8
Maize flour	(n=3)	38	46	76.3	91.8	10.0	4.9
Maize fine grits	(n=3)	38	56	76.3	113	5.6	4.7
Wheat	(n=3)	42	53	83.9	106	5.9	13.8
Maize	(n=1)	50	51	100.1	101	-	-
Barley	(n=3)	36	39	71.6	77.0	4.0	4.8
Barley	(n=3)	36	36	71.2	72.2	8.4	14.8
Shredded wheat	(n=3)	47	58	94.3	117	5.5	3.3
Shredded wheat	(n=1)	47	49	93.5	98.8	-	-
Oats	(n=6)	51	51	103	102	5.0	5.6

Table III. Matrix Spiked with T-2 and HT-2 (1:1)

Sample	*Spiking Level, Total Toxin*	*Average ppb (n=3)*		*Average Recovery,%*		*% CV*	
		T-2	HT-2	T-2	HT-2	T-2	HT-2
Organic wheat grain	100 ppb	35	50	70.6	101	4.8	5.7
Maize fine grits	100 ppb	45	53	89.9	106	8.1	9.7
Pearl barley	100 ppb	53	57	106	114	13.5	13.5
Maize flour	500 ppb	249	298	99.6	119	5.5	5.7
Oats	100 ppb	49	47	97.8	94.2	3.7	12.1

Table IV. Naturally Contaminated Barley

	Average GC-MS Result (ppb) (n=3)		*Average EASI-EXTRACT® T-2 and HT-2 Result (ppb) (n=3)*	
Reference Material	T-2	HT-2	T-2	HT-2
	26	71	19	60

Results and Discussion

When the EASI-EXTRACT® T-2 and HT-2 columns were challenged with equal amounts of T-2 and HT-2 toxins the recovery was 100% for both toxins demonstrating the antibody has equal affinity for T-2 and HT-2. The capacity of the column was also shown to be at least 1.5 µg total toxin (T-2/ HT-2 – 1:1), while the limit of detection (LOD), at a signal to noise ratio of 3:1, was 1 ppb for HT-2 and 10 ppb for T-2 using the recommended LC conditions. The limit of quantification (LOQ), defined as 3 x LOD, was 3 ppb for HT-2 and 30 ppb for T-2. Both the LOD and the LOQ could be further improved by passing a greater volume of sample extract through the immunoaffinity column.

Maize, barley, wheat, and oats extracts were spiked at 100 ppb (50 ng T-2 /g and 50 ng HT-2/g). Recoveries ranged from 71% to 117% for both toxins in all cereal extracts tested with a % CV of 3.3% to 14.8% (Table II). The results confirmed that the EASI-EXTRACT® T-2 and HT-2 columns were suitable for analysis of T-2 and HT-2 in a variety of cereal extracts.

Following the prescribed protocol, cereal samples were extracted and spiked following filtration with 100 ppb toxins (50 ng/g T-2 and 50 ng/g HT-2) or 500 ppb toxins (250 ng T-2 /g and 250 ng HT-2/g). Recoveries ranged from 71% to 119% for both toxins with a % CV of 3.7% to 13.5% (Table III). Figures 1 and 2 show the effect of including a 2% sodium chloride dilution step for the analysis of oats. This step clearly facilitates protein precipitation prior to using the immunoaffinity column, resulting in much cleaner chromatography.

Finally a barley reference material was analysed over several days using the immunoaffinity columns with LC-fluorescence (Figure 3) and results compared to GC-MS following trimethylsilyl derivatization (*6*). The LC results compared well to those obtained using GC-MS following trimethylsilyl derivatization (Table IV) and were reproducible over several days (Table V).

Conclusions

Recoveries of T-2 and HT-2 using EASI-EXTRACT® T-2 and HT-2 columns on cereal samples spiked with total T-2 and HT-2 toxins at 100 ppb and

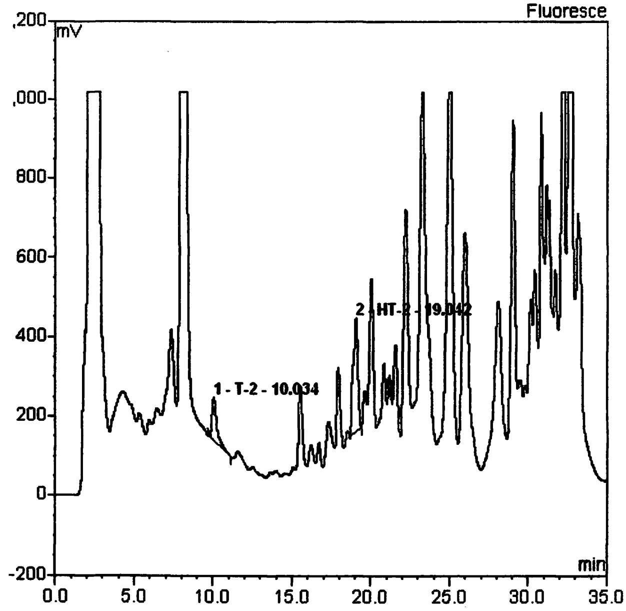

Figure 1. Oats spiked with 100 ppb total T-2 and HT-2 toxin (1:1) analysed without a 2% sodium chloride dilution.

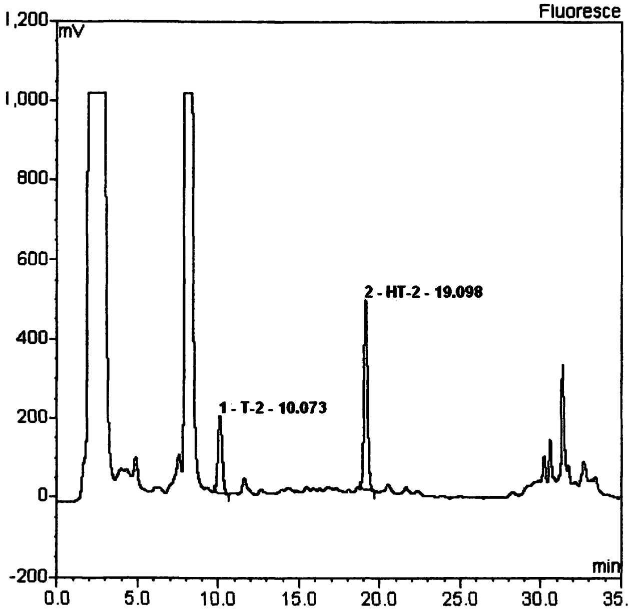

Figure 2. Oats spiked with 100 ppb total T-2 and HT-2 toxin (1:1) analysed with the inclusion of 2% sodium chloride dilution step to aid precipitation of proteins.

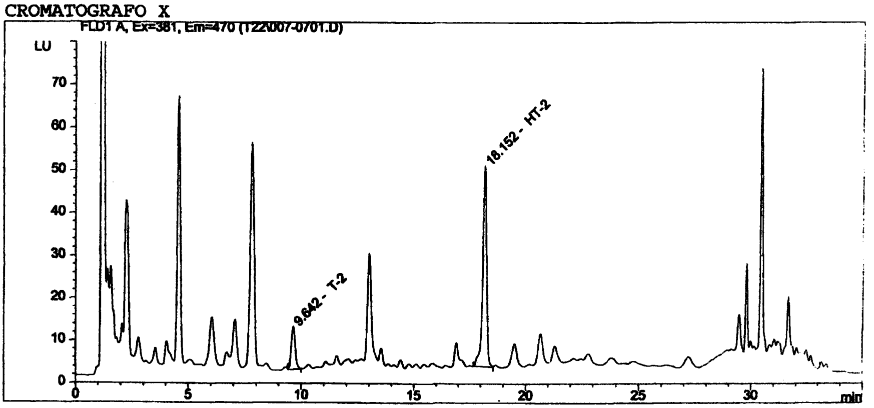

Figure 3. LC Trace of Naturally Contaminated Barley Reference Material using EASI-EXTRACT® T-2 & HT-2 Columns

Table V. Naturally Contaminated Barley: Analysed over 2 Days using EASI-EXTRACT® T-2 & HT-2 IAC Columns

Sample Type	*T-2*	*HT-2*
Sample A Day 1	22	80
Sample A Day 2	24	90
Sample B Day 1	13	33
Sample B Day 2	15	41

500 ppb were within the European Union (EU) specification of 60% to 120%. Analysis of naturally contaminated reference samples for T-2 and HT-2 also showed consistent results over two days giving a percentage relative standard deviation (% RSD) of <15%, which is again within the proposed parameters of ≤40%RSD specified by the EU. The contamination levels found using the method on naturally contaminated reference samples were also similar to those obtained using GC-MS following trimethylsilyl derivatization, confirming that the use of immunoaffinity columns with LC-fluorescence was an acceptable method for determination of T-2 and HT-2 in cereals.

The results demonstrate that the EASI-EXTRACT® T-2 and HT-2 immunoaffinity columns offer a rapid, easy-to-use method for the determination of T-2 and for HT-2 in wheat, barley, maize, and oats.

Acknowledgement

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Chapter 16

A Lateral Flow Test for Detection of Total Aflatoxin in Roasted Peanuts

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Lateral flow tests named AgraStrip® were validated to test for total aflatoxins in roasted peanuts at cut-off levels of 4 ppb, 10 ppb and 20 ppb, respectively. The test is a one-step lateral flow immunochromatographic assay based on competition immunoassay format. It is a rapid semi-quantitative method with assay results obtained in 5 min. The study assessed accuracy of testing total aflatoxins in roasted peanuts. Results indicated the tests are accurate for semi-quantitative measurement of total aflatoxins greater or less than the indicated cut-off levels in roasted peanuts.

Aflatoxins are secondary metabolites of, primarily, the fungi *Aspergillus flavus* and *A. parasiticus*. There are four principle types of aflatoxin: B_1, B_2, G_1 and G_2, which are named for their respective innate fluorescent properties and mobility in chromatograms. Aflatoxins are known as most potent chemical liver carcinogens. They can cause liver disease in animals and may cause other diseases involving other organ systems (*1*).

Aflatoxins can be found mainly in cereals, corn, peanuts, cottonseed, and nuts. Peanuts, in all geographical locations, are prone to contamination with aflatoxins in the field before and after harvest, during curing, and in storage (*2*). The reason why the incidence is more frequent in peanuts than in other

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ACS Symposium Series; American Chemical Society: Washington, DC, 2008.

agricultural commodities is not fully understood, however, it could be attributed to the fact that *A. flavus* dominates the mycoflora of peanut field soils, where the kernels develop and mature beneath the surface (*3*).

Regulations for aflatoxins in commodities and food exist in at least 100 countries; maximum tolerated levels differ greatly among countries (*4*). Proper sampling procedures are pre-requisite for obtaining reliable results in aflatoxin analysis due to the heterogeneous distribution of aflatoxins in grains and other commodities (*5*). Commonly used analytical methods for the determination of aflatoxins include thin-layer chromatography (TLC), liquid chromatography (LC), gas chromatography (GC), and immunochemical methods such as enzyme-linked immunosorbent assay (ELISA). Although these methods could give quantitative results, there are some disadvantages such as: high investment costs for equipment/instrument, skilled chemists/analysts are required, and long testing times, etc. The industry needs rapid on-site test methods to obtain results within minutes but which do not require professional training and high investment (*6*). Such rapid tests fit perfectly for analysis of aflatoxins in the peanut industry.

The immunochromatographic test, also called lateral flow test or strip test, has been used in clinical applications for years. However, its application in food safety, especially mycotoxin testing, is quite recent (*7*). The AgraStrip® test is a one-step lateral flow immunochromatographic assay based on a competition immunoassay format. Antibody-particle complex is dissolved in assay diluent and mixed with sample extract. The mixed content is then wicked onto a membrane, which contains a test zone and a control zone. The test zone captures free antibody-particle complex, allowing colored particles to concentrate and form a visible line. A positive sample containing aflatoxins greater than the cut-off level will result in no visual line in the test zone (T). Alternatively, a negative sample containing aflatoxin less than the cut-off level will form a visible line in the test zone. A line will always be visible in the control zone (C) regardless of the presence of aflatoxin.

This article reports an in-house validation study of AgraStrip® total aflatoxin test kits for the detection of total aflatoxin in roasted peanuts.

Materials and Methods

Materials

Chemicals

Aflatoxin B_1, B_2, G_1 and G_2 standards (concentrations of 10 μg/mL each) were obtained from Biopure Referenzsubstanzen GmbH (Austria). Total aflatoxin spiking standards containing aflatoxins B_1, B_2, G_1 and G_2 at a ratio of 10:1:1:1, respectively, were prepared from the four individual aflatoxin

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standards. Methanol used for sample extraction was ACS grade purchased from Fisher Scientific (UK). Chemicals used in LC methods such as KBr, acetonitrile, and HNO_3 were HPLC grade, purchased from Sigma Aldrich (USA).

Test Kits

Test kits used in the study were AgraStrip® Total Aflatoxin Test 4 ppb Cut-off (lot no. 011130-0606), AgraStrip® Total Aflatoxin Test 10 ppb Cut-off (lot no. 021130-0606), and AgraStrip® Total Aflatoxin Test 20 ppb Cut-off (lot no. 031130-0605). Each AgraStrip® aflatoxin test kit includes 24 aflatoxin test strips, 24 microwells coated with antibody-color particle complex (sealed with clear tape), 1 bottle of assay diluent, and 1 microwell holder.

Roasted Peanuts

Roasted peanuts were from USA. Peanut samples were ground with a blender and mixed until homogenous. Prior to fortification, at least four 50 g sub-portions of ground peanut samples were analyzed for the presence of aflatoxin residues using the Romer Labs in-house validated HPLC method, i.e., AflaKobra.HPLC.003. Only those containing less than 2 µg/kg of total aflatoxins were used as aflatoxin-free control, as well as for sample fortifications.

Methods

Extraction Methods

Ten g of ground roasted peanuts sample was weighed into a clean 50 mL falcon tube; 20 mL of methanol-water (70:30, v/v) extraction solvent was added into the tube, which was then sealed and vigorously vortexed for 1 min; the top layer of extract was filtered through a Whatman #1 filter paper and the filtrate used for testing.

Test Kit Assay Methods

The microwell sealer was removed and an appropriate number of microwells was placed in a microwell holder. Fifty µL of assay diluent was add to each microwell using a single channel pipette. The coated color material in the microwell was dissolved by pipetting the content up and down 5 times. Fifty µL of sample extracts was added to each microwell and the content in each well was

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was mixed by pipetting it up and down 3 times. One test strip was placed into each microwell, and allowed to develop color for 5 minutes. The test results were interpreted after 5 min.

Interpretation of Results

(1) Visual interpretation: a color line always appeared in the upper section of the test strip to indicate that the test strip was working properly. This line was the Control Line (C). A line in the lower section of the test strip indicated the test result. This line was the Test Line (T). If the results were less than the cut-off level, both T and C lines were visible. If the results were greater than or equal to the cut-off level, only C line was visible. If there was no line in control zone, the test was invalid and the sample should be re-tested by using a valid test strip (Figure 1).

(2) AgraStrip Reader: the strips were read by using an AgraStrip Reader, and the results were obtained in seconds (Figure 2). If the aflatoxin results were less than the cut-off level (negative sample), the AgraStrip Reader would display a negative sign "–". If the results were greater than or equal to the cut-off level (positive sample), the AgraStrip Reader would display a positive sign "+" (Figure 3).

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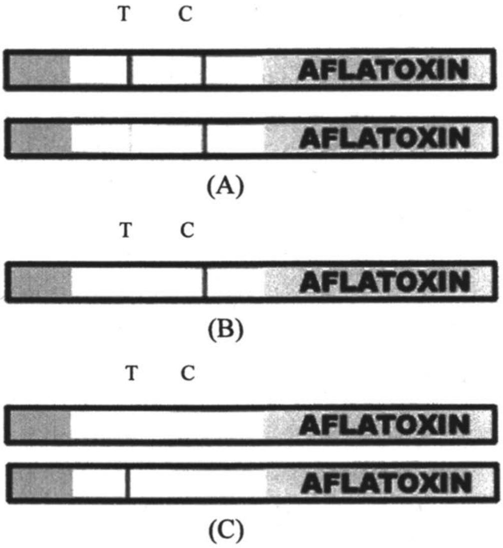

Figure 1. Visual interpretation of AgraStrip Aflatoxin test. (A) Aflatoxin less than the cut-off level: both T and C lines visible; (B) Aflatoxin greater than or equal to the cut-off level: only C line visible; (C) Invalid test: C line invisible.

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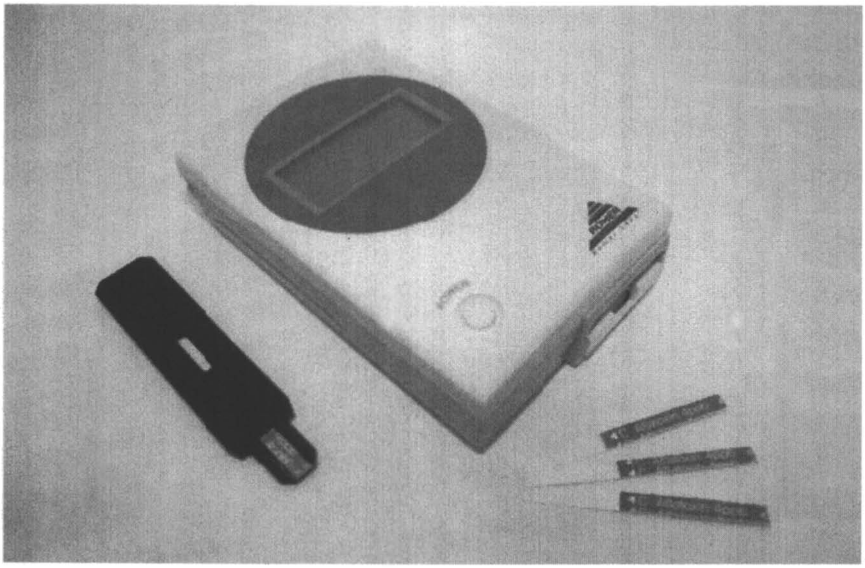
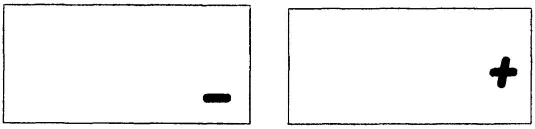


Figure 2. AgraStrip reader and strip holder



(A) (B)

Figure 3. Results display in AgraStrip Reader. (A) A negative sign "–" indicates aflatoxin less than the cut-off level; (B) A positive sign "+" indicates aflatoxin greater than or equal to the cut-off level.

LC method

An in-house validated LC method with post-column derivatization using Kobra Cell (Coring, Germany) and MycoSep® 226 clean-up column (Romer Labs Inc., USA) was used for the quantitation of aflatoxins B_1, B_2, G_1 and G_2. An Agilent 1100 series HPLC was used with binary pump G1312A and fluorescence detector 1321A (Agilent, USA). The mobile phase was water-acetonitrile-methanol (5:1:1, v/v/v). One hundred µl HNO_3 and 0.3g of KBr were added into 1 liter of mobile phase for post-column bromine derivatization with the Kobra Cell. The flow rate of mobile phase was 2 mL/min. Excitation and emission wavelengths were 360 nm and 440 nm, respectively. Sample injection volume was 100 µl. The retention times for aflatoxins G_2, G_1, B_2, B_1 were 10, 12, 14, and 18 min, respectively.

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Validation Methods

Four sets of ten-gram samples of roasted peanuts, which were confirmed to be aflatoxin-free, were spiked at total aflatoxin concentrations of 0, 2, 4, 7.5, 10, 15, 20, and 25 µg/kg. Three sets of each of aflatoxin fortified samples were extracted for AgraStrip® test kit testing. Fortification concentrations of 0, 2, 4, and 7.5 µg/kg were used for evaluating the AgraStrip® Total Aflatoxin Test 4 ppb Cut-off; fortification concentrations of 0, 7.5, 10, and 15 µg/kg were used for evaluating the AgraStrip® Total Aflatoxin Test 10 ppb Cut-off; and fortification concentrations of 0, 15, 20, and 25 µg/kg were used for evaluating the AgraStrip® Total Aflatoxin Test 20 ppb Cut-off. Each extract was analyzed in triplicate with each type of test kit by one analyst using both visual interpretation and AgraStrip reader. The fourth set of samples was analyzed using the LC method to confirm the correctness of fortification concentrations.

Results and Discussion

The control line was observed in all tests throughout the study, indicating the validity of the testing results. For the AgraStrip® Total Aflatoxin Test 4ppb Cut-off, the test line was clearly observed in the roasted peanuts containing no detectable aflatoxin. The test line was also visible for the 2 µg/kg aflatoxin spiked roasted peanuts although the color was faint. No test line was observed for the 4 µg/kg and 7.5 µg/kg aflatoxin spiked roasted peanuts, indicating these samples were positive based on the cut-off level of 4 ppb (Table I). The results from the AgraStrip reader clearly showed "–" (negative) for non-detectable and 2 µg/kg samples, and "+" (positive) for 4 µg/kg and 7.5 µg/kg samples. Hence, the visual readings were consistent with results from the AgraStrip reader.

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Table I. Results for AgraStrip® Total Aflatoxin Test 4 ppb Cut-off on Aflatoxin Spiked Roasted Peanuts

Aflatoxin Concentration in Roasted Peanuts (μg/kg)	*Number of Tests*	*AgraStrip Visual Readings*		*Results from AgraStrip Reader (+/–)*	*Result Interpretation*	*Comments*
		Test Line	*Control line*			
< 2.0	9	Visible	Visible	(–)	Negative	Valid
2.0	9	Visible (faint)	Visible	(–)	Negative	Valid
4.0	9	Non-Visible	Visible	(+)	Positive	Valid
7.5	9	Non-Visible	Visible	(+)	Positive	Valid

NOTE: the test no. of 9 for each fortification concentration is composed of three samples and testing of each sample in triplicate.

For the AgraStrip® Total Aflatoxin Test 10 ppb Cut-off, the test line was visible for the non-detectable and 7.5 μg/kg aflatoxin spiked roasted peanuts samples, although the test line was faint for the 7.5 μg/kg samples. The results indicate the samples were negative based on the cut-off level of 10 ppb. No test line was observed for 10 μg/kg and 15 μg/kg aflatoxin spiked roasted peanuts, indicating these samples were positive (Table II). The results from AgraStrip reader showed "–" (negative) for the non-detectable and 7.5 μg/kg samples, and "+" (positive) for the 10 μg/kg and 15 μg/kg samples. Results from the AgraStrip reader were consistent with visual interpretations.

For the AgraStrip® Total Aflatoxin Test 20 ppb Cut-off, the test line was clearly visible for the non-detectable and 15 μg/kg aflatoxin spiked roasted peanuts samples, showing the samples were negative. No test line was observed for 20 μg/kg and 25 μg/kg aflatoxin spiked roasted peanuts samples, indicating these samples were positive (Table III). Results from the AgraStrip reader were consistent with the visual interpretations.

LC analysis confirmed the concentrations in the fortified corn samples (Table 4). Recovery of total aflatoxin from spiked roasted peanuts by the LC method ranged from 80 – 114 %. This indicates the aflatoxin concentrations in the samples of spiked roasted peanuts used in the test kit study were accurate. Overall, the test kit results for testing total aflatoxins in roasted peanuts were consistent with the reference HPLC method.

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Conclusions

AgraStrip® Total Aflatoxin 4 ppb Cut-off, 10 ppb Cut-off and 20 ppb Cut-off Test Kits are able to test total aflatoxins in roasted peanuts at their respective detection limits.

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Table II. Results for AgraStrip® Total Aflatoxin Test 10 ppb Cut-off on Aflatoxin-Spiked Roasted Peanuts

Aflatoxin Concentration in Roasted Peanuts (µg/kg)	*Number of Tests*	*AgraStrip Visual Readings*		*Results from AgraStrip Reader (+/–)*	*Result Interpretation*	*Comments*
		Test Line	*Control line*			
< 2.0	9	Visible	Visible	(–)	Negative	Valid
7.5	9	Visible (faint)	Visible	(–)	Negative	Valid
10.0	9	Non-Visible	Visible	(+)	Positive	Valid
15.0	9	Non-Visible	Visible	(+)	Positive	Valid

NOTE: the test no. of 9 for each fortification concentration is composed of three samples and each testing of each sample in triplicate.

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Table III. Results for AgraStrip® Total Aflatoxin Test 20 ppb Cut-off on Aflatoxin-Spiked Roasted Peanuts

Aflatoxin Concentration in Roasted Peanuts (μg/kg)	*Number of Tests*	*AgraStrip Visual Readings*		*Results from AgraStrip Reader (+/–)*	*Result Interpretation*	*Comments*
		Test Line	*Control line*			
< 2.0	9	Visible	Visible	(–)	Negative	Valid
15.0	9	Visible	Visible	(–)	Negative	Valid
20.0	9	Non-Visible	Visible	(+)	Positive	Valid
25.0	9	Non-Visible	Visible	(+)	Positive	Valid

NOTE: the test no. of 9 for each fortification concentration is composed of three samples and testing of each sample in triplicate.

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Table IV. LC Confirmation Results on the Fortified Samples

Aflatoxin Concentration in Roasted Peanuts (μg/kg)	*LC Results (μg/kg)*	*Recovery (%)*
Non-detectable	< 2.0	\
2.0	1.7	86
4.0	3.2	80
7.5	8.6	114
10.0	10.7	107
15.0	16.8	112
20.0	20.7	104
25.0	23.7	95

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Chapter 17

Ethanol Extraction Method for a Rapid Test for Aflatoxin in Corn

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Methanol-water mixtures or acetonitrile-water mixtures have been used for years for the extraction of aflatoxin from corn, grains, and nuts. Many labs are concerned about the health hazard of acetonitrile and the hazardous waste disposal charges for both acetonitrile and methanol. The data in this presentation show that ethanol can be used as a substitute for methanol in a rapid method for total aflatoxin determination using immunoaffinity column chromatography with fluorescence detection (AflaTest). The ethanol extraction method meets the same performance specifications for precision, accuracy and limit of detection as the methanol extraction method. The results from the rapid method using ethanol and water extraction also correlate with an HPLC method using methanol extraction. Ethanol can then be used rather than methanol as a solvent for the rapid determination of aflatoxin thus reducing hazardous waste generation.

In Food Contaminants; Siantar, D., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2008.

Aflatoxins are naturally occurring toxins produced by *Aspergillus flavus*, *A. parasiticus*, and other *Aspergillus* fungi growing on corn, grains, nuts, spices, cottonseed, and other food products. Aflatoxin is classified by the International Agency for Research on Cancer (IARC) as a group one (proven) carcinogen along with being hepatotoxic and immunosuppressive (*1*). Aflatoxins are regulated worldwide at levels from zero to 35 ppb (µg/kg) (*2*).

VICAM's aflatoxin test kit using methanol-water (80:20, v/v) extraction has received United States Department of Agriculture - Grain Inspection, Packers and Stockyards Administration (USDA-GIPSA) Certificate of Conformance no. FGIS 2006-101 as a quantitative test kit for the determination of total aflatoxins in corn, condensed distillers solubles, corn bran, corn flour, corn germ meal, corn gluten feed, corn gluten meal, corn meal, corn soy blend, distillers dried grains, distillers dried grains with solubles, flaking corn grits, milled rice, popcorn, rough rice, sorghum, soybeans, and wheat. The objective of this study was to generate supporting data for VICAM's AflaTest® procedure to detect aflatoxin in corn for food or animal feed using immunoaffinity column chromatography with fluorescence detection while substituting ethanol for the methanol used in extraction and elution. Results generated were evaluated using performance criteria set by the USDA-GIPSA (*3*).

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Materials and Methods

Materials

Immunoaffinity columns (AflaTest®), filter papers, bromine developer solution, and fluorometer calibration standards were obtained from VICAM (Watertown, MA). Deionized water was from a MilliQ$_{plus}$ system (Millipore Corp). Ethanol was not denatured: HPLC/spectrophotometric Grade Ethanol, 200 proof (example: Sigma cat # 459828). Methanol was HPLC grade. All other reagents were ACS grade or better. A VICAM Series 4 fluorometer was used for quantitation.

Procedure

This study determined the limit of detection, accuracy, precision, and correlation with LC values. The limit of detection was determined by multiple measurements on aflatoxin-free corn. Accuracy and precision were determined using aflatoxin-free corn samples spiked at five and 20 ppb total aflatoxins (in the ratio of 10 aflatoxin B_1: 1 aflatoxin B_2: 1 aflatoxin G_1: 1 aflatoxin G_2). Correlation with HPLC values was determined by assaying four well-ground and mixed naturally aflatoxin contaminated corn samples that had previously been quantified by HPLC.

The ethanol extraction and elution method for aflatoxin quantitation is as follows. Fifty g of ground corn sample was blended for one min with 5 g of sodium chloride and 100 mL ethanol-water (80:20, v/v). The mixture was passed through a medium filter paper (VICAM part #31240) and the extract collected in a clean vessel. Ten mL of the extract was diluted with 20 mL of water and the extract was filtered again through a microfiber filter paper (VICAM part #31955) to remove particles. One mL of the extract was passed through an AflaTest® immunoaffinity column (VICAM part #12022), containing immobilized antibodies to aflatoxin, at a rate of about one drop per second. The column was washed with two one mL portions of water at a rate of about one drop per second. The aflatoxin was eluted from the immunoaffinity column by one mL of ethanol at a rate of about one drop per second and the one mL eluate collected in a glass cuvette. A developer solution (VICAM part #32010) consisting of a dilute bromine solution was added to the cuvette to enhance the fluorescence of aflatoxin B_1 and G_1. The cuvette was then read after 60 sec in a VICAM series 4 fluorometer calibrated with fluorescent calibration standards (VICAM part #33030) setting the red calibration vial to 180 and the green calibration vial to -3.0. The fluorometer readout was in parts per billion (ppb, µg/kg). The methanol extraction and elution method was the same as above with the following exceptions. The sample was blended with 100 mL methanol-water (80:20, v/v) rather than ethanol:-water. The immunoaffinity column was eluted with 1 mL methanol rather than ethanol. The fluorometer was calibrated by setting the red vial to 160 rather than 180. The change in calibration compensates for a slight difference in fluorescence of aflatoxin in methanol compared to ethanol. The green vial setting remained the same at -3.0. The methanol extraction and elution method is a modification of the AOAC International Official Method **991.31** (*4*).

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Results and Discussion

Limit of Detection

The performance evaluation standards from the USDA-GIPSA for aflatoxin kits define the Limit of Detection (LOD) as equal to the mean plus twice the standard deviation (SD) for an aflatoxin-free ground corn sample. An aflatoxin-free corn sample (less than 0.2 ppb aflatoxin) was spiked with one ppb of aflatoxin. Both the aflatoxin-free and one ppb sample were extracted and the extract assayed ten times. Results are presented in Table I. The mean plus twice the SD of an aflatoxin-free corn sample is zero ppb. The USDA-GIPSA evaluation standard for LOD is less than or equal to 3.0 ppb. The ethanol-water extraction method meets the USDA-GIPSA standard for LOD. In order to obtain an actual value for limit of detection, we have tested a corn sample spiked at one ppb aflatoxin as well. Defining LOD as the minimum level at which aflatoxin can be reliably detected, the ethanol-water extraction method can detect aflatoxin at one ppb.

Table I. Limit of Detection

Sample	*Aflatoxin-free corn*	*1 ppb Spiked Corn*
1	0	1.6
2	0	1.9
3	0	1.3
4	0	1.2
5	0	0.78
6	0	0.66
7	0	0.45
8	0	0.34
9	0	1.2
10	0	1.1
Mean	0	1.1
SD	0	0.50
% RSD	0	47%

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Accuracy and Precision

Five aflatoxin-free ground corn samples were spiked at 5 ppb and five were spiked at 20 ppb. Total aflatoxin was spiked in a ratio of 10 aflatoxin B_1: 1 aflatoxin B_2: 1 aflatoxin G_1: 1 aflatoxin G_2. Each sample extract was analyzed once. Results are presented in Table II. The USDA-GIPSA performance evaluation standards for accuracy and precision are listed in Table II. The ethanol extraction and elution method is accurate and gives 18% RSD at 5 ppb and 4.6% RSD at 20 ppb. The method meets USDA-GIPSA performance standards for both accuracy and precision.

Correlation with HPLC and Methanol Extraction

Four ground corn samples naturally contaminated with aflatoxins at approximately 5, 10, 20, and 100 ppb were extracted in duplicate using the

Table II. Accuracy and Precision

Corn Sample	*Spike Level*	*USDA-GIPSA Standards*
	5 ppb spike	
Sample 1	6.5	
Sample 2	4.4	
Sample 3	4.8	
Sample 4	4.4	
Sample 5	4.5	
Mean	4.9	2-8
SD	0.90	< 1.5
% RSD	18	< 30%
	20 ppb spike	
Sample 1	20	
Sample 2	20	
Sample 3	20	
Sample 4	19	
Sample 5	18	
Mean	19	10-30
SD	0.90	< 5.0
% RSD	4.6	< 25%

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ethanol extraction and elution procedure. Each extract was then analyzed in duplicate. These samples had been previously extracted and analyzed 21 times using the methanol extraction and elution AflaTest fluorometer method as well as 21 times by LC using the USDA-GIPSA Aflatoxin Reference Method; this is a modification of AOAC International Method **991.31** which has a methanol-water extraction and methanol elution. The data are listed in Table III. The fluorometer procedure using ethanol extraction and elution shows excellent correlation with LC results as shown by the R^2 value of 0.9966 in Figure 1. The correlation between fluorometer results performed using ethanol and those using methanol is also excellent, with an R^2 value of 0.999 as shown in Figure 2. Correlation between the ethanol-water and methanol-water extractions was closer at the 5, 10 and 20 ppb levels than at the 100 ppb level. Further samples naturally contaminated at the 100 ppb level would need to be tested to evaluate results at 100 ppb.

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Table III. Correlation of Ethanol and Methanol Methods

Method	*Sample 1 Total Aflatoxin (ppb)*	*Sample 2 Total Aflatoxin (ppb)*	*Sample 3 Total Aflatoxin (ppb)*	*Sample 4 Total Aflatoxin (ppb)*
Ethanol extraction/elution	4.4	13	24	94
	5.0	13	24	92
	5.6	7.2	22	92
	5.3	7.2	22	88
Ethanol mean (fluorometer)	5.1	10	23	92
Methanol extraction/elution, mean of 21 results (fluorometer)	4.9	9.4	24	115
LC - mean of 21 results	5.7	9.2	21	112

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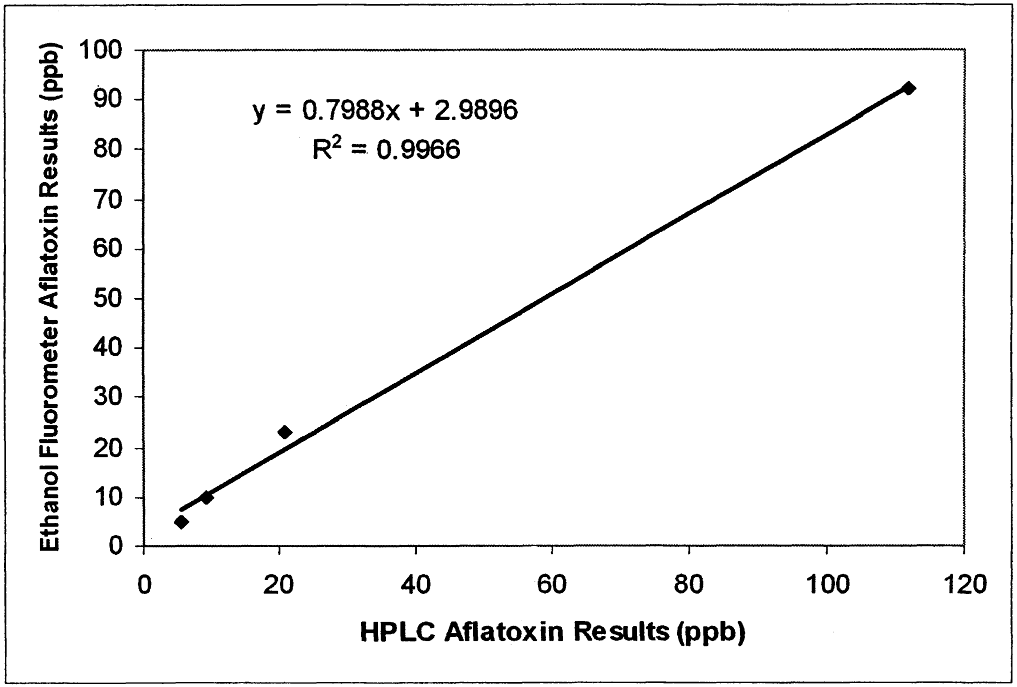

Figure 1. Aflatoxin results with ethanol fluorometer vs. LC

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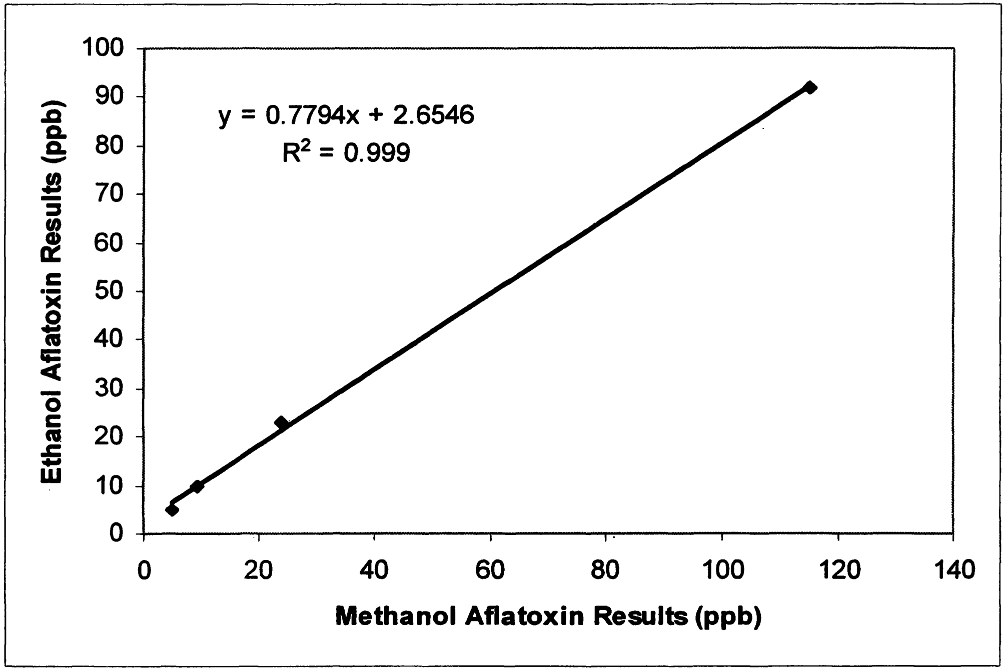

Figure 2. Ethanol vs. methanol extraction - aflatoxin fluorometer results

Conclusions

A method employing ethanol-water extraction and ethanol immunoaffinity column elution can be used instead of a methanol-water extraction and methanol immunoaffinity column elution for the determination of aflatoxins using immunoaffinity column clean up with fluorometer detection. Results using ethanol are comparable to the methanol results. Replacing methanol with ethanol for aflatoxin determination will reduce hazardous waste generation.

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Chapter 18

Highly Sensitive and Quantitative Method for Measuring Aflatoxin M_1 in Milk

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Aflatoxin M_1 (AFM_1) is a metabolite of aflatoxin B_1 found in milk of dairy cattle which have consumed feed contaminated with aflatoxin B_1. AFM_1 has been reported to be carcinogenic and hepatotoxic. The European Commission has established maximum permissible limits of 50 parts per trillion (ppt) for AFM_1 in milk. We have developed an assay for AFM_1 which meets the EU sensitivity requirements and can be performed using a simple single-well fluorometer. The method utilizes a novel "mobile bead" configuration immunoaffinity column and requires less than 40 min including sample preparation. The assay has excellent linearity (R^2 = 0.998) over a concentration range of 0-200 ppt. Precision at 25 ppt and 50 ppt was excellent with % CV below 10%. The limit of detection was 12.5 ppt.

In Food Contaminants; Siantar, D., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2008.

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Aflatoxin B_1 (AFB_1) is one of the most toxic and common natural toxins found in grains, oilseeds, spices, and tree nuts. It has significant impact on food/feed quality, human health and agricultural economics. It has been well-known that AFB_1 is a carcinogen, and that high level exposure produces an acute necrosis, cirrhosis and carcinoma of the liver in mammals (*1*). Aflatoxin M_1 (AFM_1) is one of the metabolites of AFB_1 in human and animals that consume commodities contaminated with AFB_1. It has been found in urine, milk and meats (*1, 4*). The estimated conversion rate from AFB_1 to AFM_1 in vivo is about 1-3% (*2, 3*). AFM_1 is a hydroxylated form of AFB_1, also with carcinogenic properties. Both compounds are heat-stable and react with DNA and albumin. AFM_1 and its DNA/albumin adducts have been used as biomarkers to determine the degree of human exposure (*4, 7*).

The U.S. Food and Drug Administration (FDA) has established maximum limits for aflatoxin levels in human foods and animal feeds. The current FDA guidelines allow 20 ppb of total aflatoxins in feed fed to lactating dairy cows and 0.5 ppb of AFM_1 in fluid milk (*5*). However, the regulatory levels of the European Union for aflatoxins in food and feed are much stricter. The action levels for AFM_1 in milk are 50 ppt for adults and 25 ppt for infants (*6*).

There are several methods commercially available for analysis of milk for AFM_1,, including liquid chromatography (LC), enzyme linked immunosorbent assay (ELISA), a lateral flow based strip test, and affinity column/fluorometric measurement. However, there is no affinity column/fluorometric method that can detect AFM_1 below 25 ppt in milk, although the LC method can determine as low as 5 ppt in liquid milk (*8*). VICAM recently has developed a simple, rapid, and quantitative method for AFM_1 test in fluid milk. This special AFM_1 test is highly sensitive, and can detect as low as 12.5 ppt AFM_1 in milk. It does not require high technical skill and can be used in the field.

Materials and Methods

Reagents and Affinity Column

Aflatoxin M_1 standard and HPLC grade methanol were purchased from Sigma. Aflatest developer concentrate (0.03% bromine), AflaM$_1$ $^{FL+}$ mobile resin affinity column, Aflatest-M^{TM} fluorometer calibration standard, glass cuvette, Afla M_1 4-position pump stand and fluorometer Series 4 were obtained from VICAM in this study. The Afla M_1 $^{FL+}$ column is a 3 mL plastic column packed with 250 μL of resin containing covalently bound antibody of anti-AFB_1.

Sample Preparation and Cleanup

If the milk is homogenized, for example purchased from a grocery store, it can be used directly for analysis. However, raw milk from a dairy farm is usually not clear and centrifugation is needed. Centrifuge approximately 50 mL milk at 2000 x g for 10 min. Carefully take the skim portion (bottom layer) of the milk for analysis without disturbing the top (fat) layer. Forty mL of skim milk is needed for each affinity column.

Column Preparation

The Afla M_1 $^{FL+}$ column is a mobile resin column. Make sure that the level of resin inside the column is horizontal. If the resin is not horizontal, gently tap the lower side of the column several times with a finger to level the resin.

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Column Chromatography

Pass 40 mL skim milk completely through the Afla M_1^{FL+} affinity column at a rate of about 1-2 drops/sec until air comes through column. Remove the Afla M_1^{FL+} column from the loading syringe barrel. Remove the top frit from the Afla M_1^{FL+} column using the Frit-picker.

Fill the Afla M_1^{FL+} column headspace with methanol-water (10:90, v/v). Fit the Afla M_1^{FL+} column to a clean glass syringe barrel. Fill the glass syringe barrel with 10 mL methanol-water (10:90, v/v), which is passed through the column at a rate of about 2-3 drops/sec until the level of solvent is about 3 cm above the top of the resin bed.

Remove the column from the syringe barrel and cap it with the cap that comes with the column. Invert the column 10 times until resin beads are completely washed from the bottom frit. Remove the top cap from the column. Fill the column headspace with methanol-water (10:90, v/v) and place the column back onto the syringe barrel. Fill the glass syringe barrel with 10 ml methanol-water (10:90, v/v) and pass it through the column at a rate of about 2-3 drops/sec. Repeat this step once more until air comes through the column.

Elute the AFM_1 from the affinity column with 1 mL methanol-water (80:20, v/v) at a rate of about 1 drop/sec until air comes through the column, collecting the eluate in a cuvette. Add 1.0 mL of AflaTest developer concentrate diluted 1:10 with water to the eluate in the cuvette. Mix well, place the cuvette in a calibrated fluorometer and read it.

Results and Discussion

Limit of Quantitation

In this study, the limit of quantitation was defined as the smallest amount of AFM_1 which is reproducibly and accurately detected. Raw milk samples were collected from two different dairy farms, and were determined to be AFM_1-free by LC analysis. The AFM_1-free milk samples were spiked with AFM_1 (Supelco, PA) at 0, 12.5, and 25 ppt. Two independent experiments were done on two different days. The results are presented in Table I.

Two AFM_1 spiking levels, 12.5 and 25 ppt in raw milk, were examined; the average fluorometer readings were 12.4 and 28.3 ppt, respectively. The percent coefficients of variation (CV) for 12.5 ppt and 25 ppt levels were 34.7% and 9.2% respectively, suggesting that the limit of quantitation for this test is at or below 12.5ppt

Linearity

Linearity was determined using AFM_1-spiked raw milk samples ranging from 0 ppt to 200 ppt. Individual data points are shown in Table II. The percent coefficient of variation was less than 10% in all spiked levels except for the 50 ppt level. The graph below shows the linear regression analysis equation for the amount of AFM_1 measured by fluorometer *vs* the amount spiked in raw milk.

The linear regression equation is $Y = 1.037x + 0.246$. The correlation coefficient (r) of 0.999 from the above linear regression equation indicates excellent linearity between the AFM_1 spiked level and flurometer reading.

Table I. Fluorometer reading in AFM_1-free and -spiked raw milk

	0 ppt spike	12.5 ppt spike	25 ppt spike
	0	14	28
	0	20	28
	0	15	31
	5.5	8	30
	0	10	29
	0	12	29
		8	24
			32
			30
			25
			25
Average reading	0.9	12.4	28.3
SD	2.2	4.3	2.6
% CV	-	34.7	9.2

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Table II. AFM$_1$ Spiking Level vs Fluorometer Reading in Milk

Spike level (ppt)	1	2	3	4	Mean	SD	CV (%)
0	0	0	0	0	0	0.0	0
12.5	12	10	12	11	11.5	1.0	8.7
25	23	25	27	27	25.4	1.7	6.6
50	65	53	59	61	57.6	6.1	10.5
100	110	100	98	95	100.6	5.6	5.6
200	200	210	220	210	208	8.4	4.0

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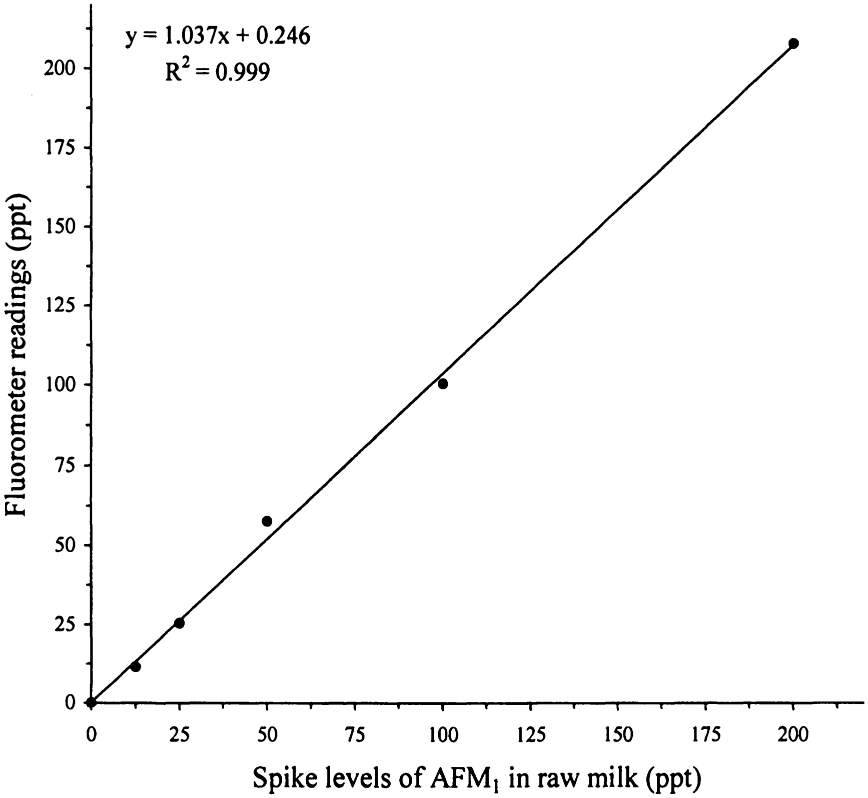

Figure 1. Dose response of AFM$_1$ spiking vs fluorometer reading

Correlation Between LC and Fluorometer Results

Naturally contaminated milk samples were collected and analyzed by LC and the VICAM fluorometer method. As shown in Figure 2, the correlation coefficient of $r = 0.96$ indicates a strong positive correlation between LC and VICAM fluorometer results, suggesting that VICAM fluorometer AFM$_1$ test is an excellent predictor of measuring AFM$_1$ in milk.

Conclusion

The VICAM AflaM$_1$ test in milk involves specific antibody-based affinity column isolation, AFM$_1$ derivatization with bromine and fluorometer reading. The intensity of fluorescent signal directly reflects the amount of AFM$_1$ in the milk sample. The test is straightforward, easy to run, highly sensitive, and

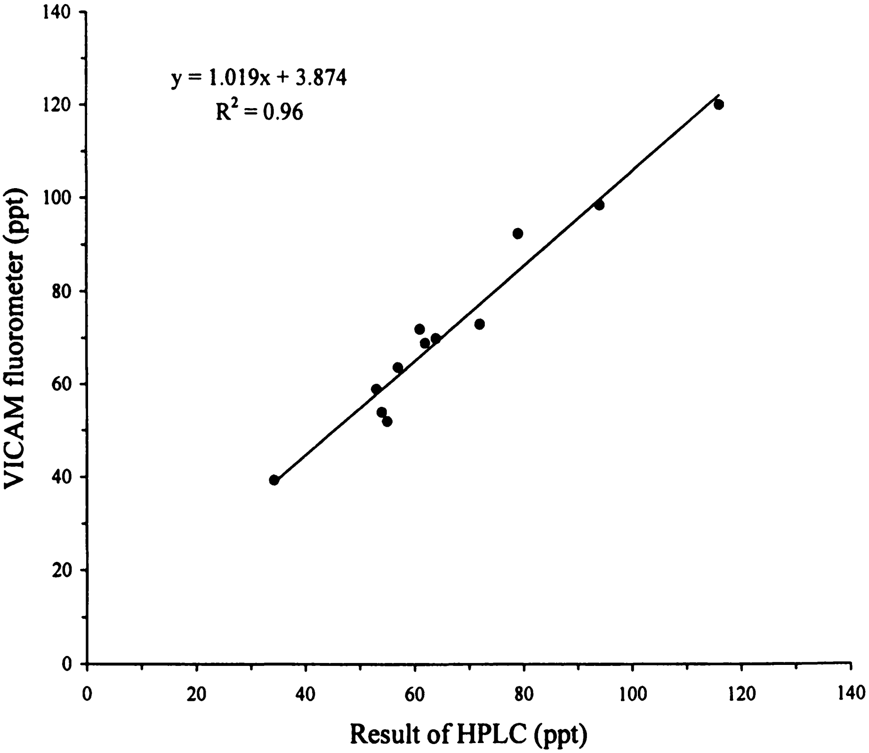

Figure 2. Naturally AFM$_1$-contaminated raw milks analyzed by LC and VICAM fluorometer method

quantitative. The result is accurate and comparable to that for LC. It does not require high technical skill and can be used in the field.

Acknowledgement

Partial data (Figure 2) from this study were generated by Drs. S. Tenti and P. Berzaghi in the Dept. of Animal Science, University of Padova, Italy.

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Chapter 19

Lateral Flow Quantitative Method for the Detection of Mycotoxins

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Lateral flow methods have been developed for the quantitative detection of mycotoxins. The method for aflatoxin detection (5-100 ppb) in corn has received GIPSA approval.The general procedure is as follows: (i) ground sample is extracted with solvent; (ii) 100 µL portion of extract is added to 1 mL dilution buffer; (iii) 300 µL portion of the diluted extract is added to a lateral flow test strip; (iv) test strip is incubated for 10 min at 45 °C and (v) reflectance reader provides a numerical result from 0 to 150 ppb by comparing the binding intensities of 2 test lines and control line. A quantitative method for zearalenone (50-1000 ppb) in corn also has received GIPSA approval. Initial in-house data are available on quantitative lateral flow methods for the detection of deoxynivalenol in wheat and ochratoxin A in wine and grape juice.

Mycotoxins are naturally occurring substances produced by mold that are harmful to human and animal health (*1-3*) Action levels have been set by the United States Food and Drug Administration (FDA) for aflatoxin in grains and feed and it is mandatory to test for aflatoxin in all corn exported from the United States. The United States Department of Agricultural Grain Inspection, Packers and Stockyards Administration (GIPSA) has established criteria for rapid test methods for aflatoxin, deoxynivalenol (DON), and zearalenone for quantitative testing, and also criteria for approving qualitative mycotoxin test kits. The lateral flow format is considered a rapid user-friendly method which has traditionally been used as a qualitative method to give a positive or negative result (*4*). There are several lateral flow qualitative methods which have received approval from GIPSA (*5*). The Charm ROSA Aflatoxin (Quantitative) and Charm ROSA Zearalenone (Quantitative) are the first lateral flow quantitative tests approved by GIPSA.

In Food Contaminants; Siantar, D., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2008.

The Charm ROSA (Rapid One Step Assay) system was initially developed for testing antibiotics in milk (*6*). The test system includes an incubator, reader and the lateral flow test strip. The methods require detection at specified tolerances/safe levels for a family of analytes, such as beta-lactams. The Charm ROSA beta-lactam test uses a biological receptor where the sensitivity of a beta-lactam to the receptor does not necessarily match the defined tolerance/safe level. Therefore in this test method the sensitivity of some of the analytes was adjusted to target detection at their tolerance/safe level (*6*). For example, the sensitivity of cephapirin is adjusted from 4 ppb towards its tolerance/safe level of 20 ppb by adding a monoclonal antibody specific for cephapirin. The ability to adjust test sensitivity to the tolerance level prevents the unnecessary destruction of milk while ensuring that the milk supply does not have a beta-lactam above the FDA established tolerance/safe levels.

The Charm ROSA quantitative tests use a test zone, a control zone, and antibody-labeled gold beads. The test zone, generally consisting of two test lines, captures antibody-labeled beads that are not bound by the target analyte. The control zone captures both bound and unbound antibody-labeled beads. In a negative sample, colored beads bind strongly to the test zone allowing only a small fraction of beads to bind to the control line. In a sample containing the target analyte there is less binding of beads to the test zone and subsequently more binding to the control line. A calibration curve is generated using a reader to compare the relative intensities of test and control lines as a function of analyte concentration. The reader includes a calibration curve to generate a concentration of analyte in the assayed strip. The quantitative tests for aflatoxin, deoxynivalenol, and zearalenone use two test lines and one control line. The ochratoxin A test for wine is a quantitative test that has one test line and one control since the defined detection range is only from 0 to 10 ppb.

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Materials and Methods

All lateral flow test kits and equipment (45 °C ROSA-M incubator and ROSA-M Reader) were manufactured at Charm Sciences, Inc. (Lawrence, MA). Certified grain samples were purchased from Trilogy Laboratories (Washington, MO). Mycotoxin standards were purchased from Trilogy Laboratories or from Sigma (St. Louis, MO). Methanol was of analytical grade or higher and purchased from Sigma. Minisart RC 15 filters were purchased from Sartorius (Hannover, Germany).

Extractions: For aflatoxin and zearalenone testing, samples were extracted using methanol-water (70:30, v/v). For official GIPSA testing, 50 g of a ground sample and 100 mL methanol-water (70:30, v/v) are blended or shaken. The sample is allowed to settle. If particles are present the extract is filtered or centrifuged. For testing aflatoxin in soybeans and distillers dried grain, 150 mL

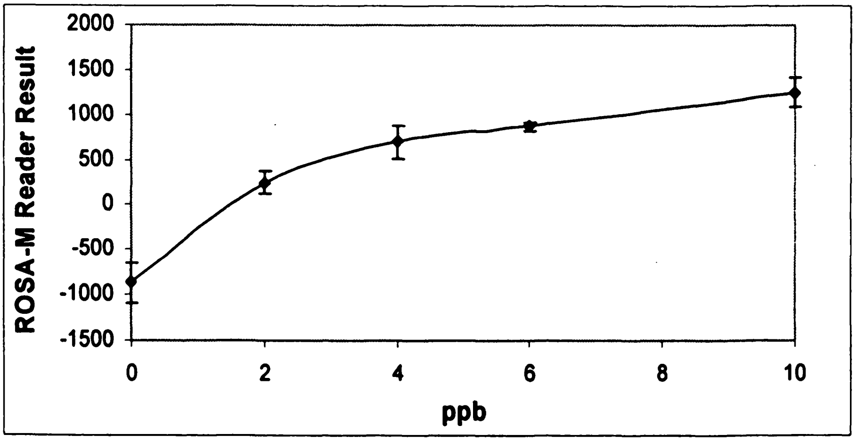

Figure 1. ROSA Ochratoxin (Quantitative) test results for red wine spiked with ochratoxin A standard. Each data point is the average of 8 test results with error bars representing one standard deviation about the mean.

of methanol is used to extract the 50 g sample. For deoxynivalenol testing, 50 g of a ground sample and 250 mL of water are blended or shaken. No extraction is required for testing wine or grape juice for ochratoxin A

Diluted Extract Preparation: For the aflatoxin, deoxynivalenol, and zearalenone tests a 100 µL portion of the extract is added to a 1 mL of dilution buffer. For aflatoxin testing in corn flour, corn meal, corn/soy blend, flaking corn grits, popcorn, and wheat a precipitate can form, which requires the mixture to be drawn into a syringe and passed through a Minisart RC 15 syringe filter. Zearalenone testing also requires the diluted extract to be syringe filtered. For ochratoxin A testing, 300 µL of the liquid sample is added to 2 mL of the ochratoxin dilution buffer.

Assay Procedure: Label test strip with sample identification and open incubator lid. Place test strip in incubator with flat side up. Peel back tape and slowly pipet 300 µL of the diluted extract into the sample compartment. Reseal tape, repeat for additional samples, and then incubate for 10 min. Remove strip and read in the ROSA-M Reader on the appropriate assay channel and matrix.

Results

The USDA/GIPSA program notice FGIS-PN-04-15 provides the performance criteria for quantitative aflatoxin tests (*7*). The program notice requires assays be run on naturally-contaminated corn samples by the test method and by LC. The results for each level must be within the defined range

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for the kit to pass. Results from an independent laboratory testing the ROSA Aflatoxin (Quantitative) lateral flow assay and LC are shown in Table I. The results are an average of 21 independent corn samples with naturally-contaminated aflatoxin levels at around 0, 5, 10, 20, and 100 ppb. Additional matrices such as corn flour, corn meal germ, corn gluten meal, corn screenings, corn soy blend, cracked corn, distillers dried grains, flaking corn, corn grits, milled rice, popcorn, rough rice, sorghum, soybeans, and wheat were also tested using fortified samples and passed specifications.

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Table I. ROSA Quantitative Lateral Flow Test Comparisons to HPLC

Mycotoxin	*LC ppb (%CV)*	*ROSA ppb (%CV)*
Aflatoxin	< 1 (NA)	1.1 (29%)
	5.7 (9%)	6.0 (26%)
	9.2 (12%)	10.0 (18%)
	21 (11%)	24 (20%)
	119 (7%)	113 (18%)
Zearalenone	0 (NA)	0 (NA)
	105 (10%)	121 (10%)
	265 (6%)	252 (9%)
	1028 (4%)	951 (11%)
DON	< 100 (NA)	0 (NA)
	700 (14%)	628 (15%)
	1100 (9%)	1144 (14%)
	1930 (4%)	2031 (11%)
	5100 (8%)	5256 (9%)

NOTE: NA is not applicable

COMMODITY: Naturally-contaminated corn was use to compare performance of aflatoxin and zearalenone test methods and naturally-contaminated wheat for DON test methods.

The detection range required for zearalenone is from 0 to 1000 ppb (*8*). Table I shows the comparison of the ROSA Zearalenone (Quantitative) assay results and LC with naturally-contaminated zearalenone in corn at about 0, 100, 250, and 1000 ppb. The results for the zearalenone tests are well within kit specifications. The reader measures the line intensities of the test lines (T1 and T2) and control line (C), determines the summed difference in intensity of each test line relative to the control line as a reader result, and calculates a concentration using the calibration curve of the test assay (*9*). As the analyte concentration in the sample increases there is a corresponding decrease in the intensities of the two test lines and a subsequent increase in the binding to the control. For example, at 1027 ppb there is a 75% inhibition in the intensity at T1, a 40% inhibition in the intensity at T2 and a 115% increase in the intensity at the control line. These changes in line intensity correlate to the quantitative result of the ROSA test kit.

Some preliminary in-house results for the Charm ROSA DON (Quantitative) test in comparison with HPLC certified samples can be seen in Table I. The detection range required for DON testing is from 0 to 5 ppm. This test uses a water extraction of the sample at a ratio of 5 parts water to one part sample.

The Charm ROSA Ochratoxin A (Quantitative) Test for wine and grape juice has a detection range from 0 to 10 ppb. Due to the narrow detection range the test format consists of one test line and a control line. White or red wine samples were spiked with ochratoxin A over a range of 0 to 10 ppb and run on the lateral flow test method. An initial titration curve for spiked red wine is presented in Figure 1, where the result is the difference in intensity between the control line and test line. Each point is the average of 8 test strips. The overall range from 0 to 10 ppb is approximately 2000 intensity points as measured by the reader. The spread from 0 to a 2 ppb spiked red wine sample is 1000 intensity points indicating the ability to detect at the 2 ppb European Union level of concern for ochratoxin A in wine.

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Summary

Lateral flow quantitative test methods were developed for aflatoxin, zearalenone, deoxynivalenol (DON), and ochratoxin A. Both aflatoxin and zearalenone have received GIPSA certification as quantitative methods for the detection of the mycotoxins. The Charm ROSA lateral flow quantitative method combines the ease of use of a lateral flow method with the precision of a quantitative method over the defined concentration range. After the extraction step and a sample dilution step the diluted sample is added to the strip, incubated, and then read in a calibrated reader. The method uses test and control zones to capture gold beads with a reflectance reader capable of measuring the intensity of the gold beads bound to each line (*9*). The relative intensity of the control line in comparison to the test line intensities is used to calculate the amount of analyte in the sample. For the corn samples naturally contaminated with zearalenone, the inhibition of intensity at the first test line (T1) for 104 ppb, 264 ppb, and 1027 ppb is 32%, 51%, and 75%, respectively, and at the second test line (T2) the inhibition is 3%, 15%, and 40%, respectively. The line intensity at the control line also changes in comparison to the zero value and for 104 ppb, 264 ppb, and 1027 ppb naturally-contaminated zearalenone samples the control line intensity increase is 70%, 84%, and 115%, respectively. For ochratoxin A the test uses one test line and one control and determines a quantitative value over a 0 to 10 ppb detection range.

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Chapter 20

Bioinformatic Tools, Resources, and Strategies for Comparative Structural Studies of Food Allergens

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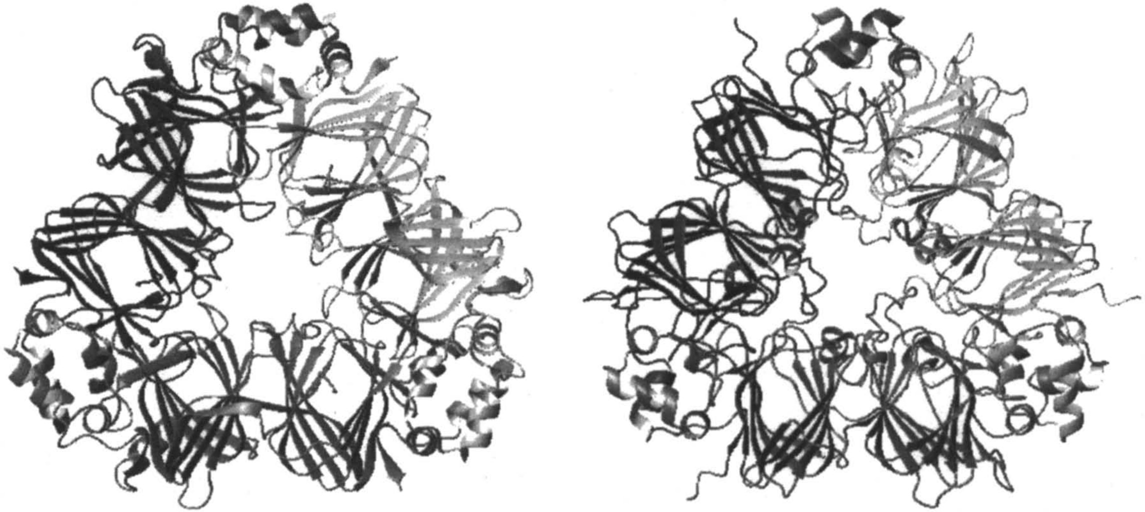
Legume seed-storage proteins, such as those from soybean and peanut, represent an important source of protein in the human diet. While generally well tolerated, these proteins also represent a potentially serious allergenic threat to many individuals. Despite high sequence similarity of peanut allergens to their relatively non-allergenic soy and kidney bean counterparts, it is not well understood why peanuts elicit such an acute allergenic response. We have employed a number of bioinformatics tools and strategies to further investigate the relationship between allergenicity and the sequence, fold, and three-dimensional structures of legume seed-storage proteins. We mapped and compared multiple features including sequence conservation amongst protein families, physical location of IgE epitopes, and residues critical for IgE binding on known and modeled protein structures. These comparisons provide for a better understanding of the relationship between legume protein structure and allergenic response in the human population so that novel legume varieties with reduced allergenicity can be identified.

In Food Contaminants; Siantar, D., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2008.

Peanut allergy (PA) has become increasingly common. Recent studies demonstrate that the prevalence of PA has doubled in 10 years and is present in approximately 1.3-1.5% of the U.S. population (*1, 2*). Peanut is the most common cause of food-induced anaphylaxis and is the single most common cause of fatal anaphylaxis (*3-5*). Approximately 1% of preschool age children are sensitized to peanut.These individuals are at risk of life-threatening anaphylactic reactions following accidental exposure to peanut protein. Currently there are no reliable means by which to stratify patients with respect to risk of severe anaphylaxis, nor are there reliable pharmacologic means by which to prevent anaphylaxis resulting from accidental peanut exposure. The only established method to prevent an allergic and potentially deadly reaction to peanut is allergen avoidance.

In this chapter, we describe the analysis and comparison of the peanut allergen proteins Ara h 1 and Ara h 3 with less allergenic counterparts from other legume species. We present a general series of bioinformatics analyses, tools, resources, and strategies that can be applied to comparative studies of other food allergens. Furthermore, we identify sequence and structural cues that may help us understand the structural components of the allergens that contribute to the severity and long-term duration of peanut allergy.

Due to the severity and increasing incidence of peanut allergy, much attention has been given to the allergenic proteins in peanut.The major allergens, Ara h 1, Ara h 2 and Ara h 3, are seed-storage proteins and have been identified (*6-9*), purified, and biophsically characterized (*10-14*). Importantly, several IgE binding epitopes have been identified (*15-17*). The recombinant proteins have been previously cloned and expressed (*15-17*) for *in vitro* studies. Other legumes such as soy, peas, and beans contain seed-storage proteins that are highly homologous (or similar) in sequence and in structure to the peanut allergens and although some individuals develop allergies to legumes other than peanuts, they outgrow the allergies and rarely have serious, life-threatening reactions. Considering the great progress that has been made in isolating peanut allergens and their recombinant counterparts, very few studies have been devoted to understanding the structural and functional similarities and differences between these allergens and their less allergenic homologs (Figure 1) (*18, 19*). Legume seed-storage proteins including those from soybean, kidney bean and peanut belong to the cupin superfamily. This functionally diverse superfamily contains proteins that also serve as enzymes, transcriptional regulators, and germins. The 7S and 11S seed-storage proteins share a common double-stranded β-helix or "jelly roll" fold extensively reviewed by Mills and co-workers (*23*). The 7S globulins are trimeric with subunit masses ranging from 40 to 80 kDa, contain a number of variable glycosylation sites and are held together by non-covalent protein-protein interactions. The 11S globulins exist as a mixture of trimers and/or hexamers with subunit masses ranging from 50 to 60 kDa, and are also held together by non-covalent interactions. The subunits are comprised of an N-



Kidney bean

Soybean β-conglycinin

Figure 1. X-ray crystal structures of the kidney bean phaseolin (PDB ID 2PHL) (20) and the soybean β-conglycinin (PDB ID 1FXZ) (21) seed-storage proteins. Each structure consists of three identical subunits (although combinations of isoforms are observed) arranged about a central axis. All protein structures and models were visualized and rendered using MOLMOL (22).

terminal "acidic" polypeptide and a C-terminal "basic" polypeptide, formed through post-translational cleavage. For the 11S globulins, the acidic and basic chains are stabilized by an intermolecular disulfide bridge. Interestingly, despite low sequence similarity of the 7S and 11S globulins, their three-dimensional structures can virtually be superimposed.

With the long-term goal of improving the nation's nutrition and health and enhancing the safety of the nation's peanut supply, we are investigating the structure and function of legume seed-storage proteins. We think that a better understanding of subtle differences between seed-storage protein structures may explain the variable allergenic profile of peanuts and other legumes. Comparative structural analysis of peanut protein structures with their closely related, yet relatively hypoallergenic counterparts from legumes will allow us to discover and understand key structural differences and help us to identify peanut cultivars that elicit a reduced allergenic response. Furthermore, this will assist in efforts to formulate specific "vaccines" from recombinant peanut sources, enabling clinically important immunological approaches to treat peanut allergy in humans (*24-29*).

The study of protein structure using bioinformatic and computational approaches is an exciting and increasingly successful, but often intimidating, aspect of biomolecular structure and function projects. Claverie and Notredame note in *Bioinformatics for Dummies* (*30*), that "Bioinformatics is nothing but good, sound, regular biology appropriately dressed so that it can fit into a computer," and that "Bioinformatics is about searching biological databases, comparing sequences, looking at protein structures, and more generally, asking biological questions." However, a quick search of the World Wide Web results in literally hundreds of protein sequence and structure databases and analysis tools each with their own advantages and disadvantages. Typically resources are developed independently over several years by many different authors. Furthermore, most tools require a high degree of manual data manipulation and management, and there is a relative dearth of "data pipelines" available to link data with multiple analysis routines and tools. While efforts to simplify and automate data analyses between applications is being pursued by both industry and academia, the use of freely available bioinformatics resources and tools to conduct comparative protein analyses can be dauntingly complex even to the seasoned experimentalist.

Nonetheless, the use of bioinformatic tools, resources, and strategies for cursory comparative structure analysis can be performed by any experimentalist with access to a computer and the internet. Some more advanced applications, such as those to model protein dynamics, require specialized programs and computational resources, but rudimentary comparative analyses are relatively straightforward and easy to access. The succession of analyses for protein sequence and structure comparison which we have used in our studies of peanut allergen structure can be broken down into a number of discrete steps:

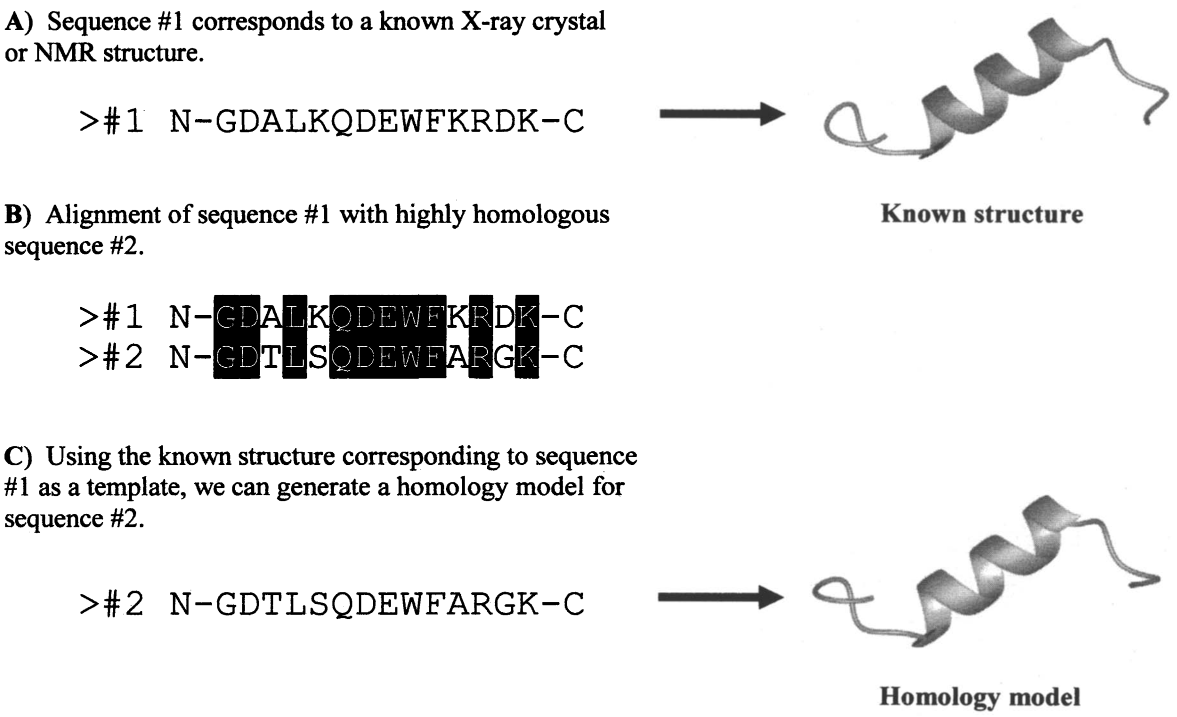
1. Initial protein database searching.
2. Protein sequence and structure database building.
3. Multiple sequence alignment and annotation.
4. Homology model generation and refinement.
5. Structure comparison and alignment of known and homologous protein structures.
6. Annotation structure alignments with pertinent biophysical data.
7. Use of *ab initio* predictive computational methods.

Generally, the first three steps are relatively straightforward, and allow for a quick and reasonably accurate assessment of the degree of similarity between two or more protein sequences. Ideally, there will exist either X-ray crystal structures or NMR-derived structures of the proteins of interest for subsequent comparison and annotation. In the event that only a few (or one) of the proteins of interest have structures available, the analyses can become somewhat more complicated. If there is sufficient amino acid sequence identity (~40-99% over the length of the protein) between a protein of interest (the target) and an available protein structure, it is possible to model the target structure using one of several freely available homology modeling servers (SWISS-MODEL (*31-33*), 3D-JIGSAW (*34-36*), Phyre (*35, 37*)). In general the homology modeling algorithms are good at fold recognition, but perhaps not the details of the structure. If there is no structure data available for the protein of interest, then *ab initio* structure prediction may be employed to determine a hypothetical structure. The field of protein structure prediction is still in its infancy, however, and while progress in the development of prediction algorithms is slowly being made, in the absence of experimentally derived structural homologues, *ab initio* structures are purely theoretical, conceptual representations of what the protein may look like.

Studies and Results

Comparative Studies of Ara h 1

Our comparative structural studies of the peanut allergens Ara h 1 and Ara h 3 with their less-allergenic counterparts is founded on the concept that similar amino acid sequences should possess similar three dimensional shapes, functional surfaces and binding sites (Figure 2). As a corollary, differences detected may impart altered structure/function relationships that impact allergenic response. It is important to note that the objectives of our studies are not to simply construct homology models of the various peanut allergens. Recently there have been several peanut allergen homology models have been



A) Sequence #1 corresponds to a known X-ray crystal or NMR structure.

```
>#1 N-GDALKQDEWFKRDK-C
```

B) Alignment of sequence #1 with highly homologous sequence #2.

```
>#1 N-GDALKQDEWFKRDK-C
>#2 N-GDTLSQDEWFARGK-C
```

C) Using the known structure corresponding to sequence #1 as a template, we can generate a homology model for sequence #2.

```
>#2 N-GDTLSQDEWFARGK-C
```

Figure 2. General conceptual foundation of comparative structural studies of proteins.

published (*10, 17, 19, 38, 39*). Rather, we used comparative structural analyses across several legume species and protein classes to provide clues regarding allergen structure that can serve as a basis from which to find peanut cultivars with reduced allergenicity.

Initial Database Searching, Database Building

Our first step in comparative studies of Ara h 1 (a 7S vicilin) begins with a search of available protein sequence databases to identify representative sequences. A number of databases are each equally suitable for these searches including those from the National Center for Biotechnology Information (NCBI: www.ncbi.nlm.nih.gov), the Universal Protein Resource (UniProt: www.pir.uniprot.org) which combines information from Swiss-Prot (the main resource on curated annotated protein sequences operated by the Swiss Institute of Bioinformatics and the European Bioinformatics Institute), the Allergen Database for Food Safety (ADFS: http://allergen.nihs.go.jp/ADFS/) operated through the Division of Biochemistry and Immunochemistry of the National Institute of Health Sciences in Japan, and the Structural Database of Allergenic Proteins (SDAP: http://fermi.utmb.edu/SDAP/) web resource located at the University of Texas Medical Branch in Galveston, Texas (*40, 41*).

A keyword text search for "Ara h 1" of the UniProt database yields three protein entries, two of which are from peanut. Accessions "P43237" and "P43238" represent recombinant peanut protein sequences that bind to serum IgE from patients with peanut sensitivity (*42*). Selection of P43237 brings up a wealth of information including date of deposition, the submitters' names, origin of the protein, literature citations, predicted structural characteristics (presence of a potential N-terminal signal sequence peptide and glycosylation sites), and the protein's primary amino acid sequence. UniProt also provides links to structural information for the similar canavalin protein from Jack Bean deposited at the Protein Data Bank (PDB: www.rcsb.org). Likewise, a keyword search for "Ara h 1" of the NCBI database yields 16 entries, three of which are associated with the peanut Ara h 1 allergen. Two of the three have the same Accession Numbers for entries at the UniProt site (P43237 and P43238) plus an additional entry (AAL27476) also isolated from a peanut genomic clone (*43*). It is useful at this point to begin collecting relevant sequences in FASTA (*44, 45*) format, and carefully comparing the sequences for potential irregularities among the sequences. Comparison of the three collected peanut Ara h 1 sequences reveal that they are essentially identical with the exception of a handful of short insertions/deletions. Because the P43237 entry was associated with experimental evidence of binding to the serum IgE of peanut sensitive individuals, this particular sequence was used in our subsequent analyses.

Sequence Comparison, Alignment, and Annotation

With a representative Ara h 1 sequence in hand, we searched the publicly available databases for similar protein sequences. Our ultimate goal here is to produce an alignment of our Ara h 1 query sequence with sequences of other proteins that have a high degree of similarity, but are relatively hypoallergenic. Furthermore, we wish to find those less-allergenic counterparts that also have available structure coordinates for additional structure-based analyses. Again, while there are a number of web resources and databases available, we used those available at the NCBI (www.ncbi.nlm.nih.gov/BLAST/) to conduct a standard protein-protein BLAST (blastp) based database (non-redundant) search (*46-48*). BLAST (Basic Local Alignment and Search Tool) and related algorithms such as PSI-BLAST, are perhaps the most popular bioinformatic tools used today. Submission of the P43237 Ara h 1 sequence to the NCBI BLAST server in December 2006 returned a distribution of over 250 BLAST hits on the query sequence. Within the top hits there are three similar proteins that have structure entries in the Protein Data Bank (a soybean beta-conglycinin (glycinin, PDB entries 1UIK (*49*), 1IPK, 1IPJ (*50*) and 1UIJ (*51*)), a 8s-alpha globulin seed-storage protein from mungbean (vicilin, PDB entry 2CV6 (*52*)) and a phaseolin from kidney bean (vicilin, PDB entry 2PHL (*20*)). Because the proteins from mungbean and kidney bean belong to the same class as Ara h 1 (vicilin) these sequences were saved in FASTA format and used for subsequent multiple sequence alignment and analysis.

ClustalW is a popular program for generating multiple sequence alignments, and again, there are a number of multiple sequence alignment resources freely available on the World Wide Web (*53, 54*). For our analyses, we used the clustalW multiple sequence alignment resource available at the European Bioinformatics Institute (www.ebi.ac.uk/clustalw/). Sequences (in FASTA format) for Ara h 1 (NCBI Accession P43237), mungbean (NCBI Accession 2CV6A), and kidney bean phaseolin (NCBI Accession 2PHLA) were submitted using the default submission parameters. The subsequent multiple alignment file was then uploaded to the ESPript (Easy Sequencing in Postscript) server (*55*) (http://espript.ibcp.fr/ESPript/ESPript/) using a Risler Similarity Global Score of 0.7. The resulting clustalW multiple sequence alignment is shown in Figure 3.

It can be seen that the sequence for Ara h 1 contains an N-terminal "leader sequence" of approximately 150 amino acids that is not present for the proteins from mungbean or kidney bean. According to the works of De Jong and Wichers (*56, 57*), this N-terminal leader section is cleaved off *in planta* from the mature Ara h 1 protein. While physically separated from the mature Ara h 1 allergen, this N-terminal leader sequence (or at least a portion of it) is still present in peanut and contains three of the 21 reported allergenic epitopes (*17*). We will return our attention to this N-terminal leader sequence later in the Chapter.

Further inspection of the multiple sequence alignment reveals that there is a relatively high degree of identity between the three sequences. When the Ara h 1 N-terminal leader fragment is not considered, 151 residues out of 459 (33%) are identical when compared to mungbean and kidney bean (423 and 397 amino acids in length respectively). Furthermore, 98 additional residues are of similar size and hydrophobicity to mungbean and kidney bean. Therefore over 50% of the amino acids for the mature Ara h 1 protein are either identical or similar to those from their closely related counterparts.

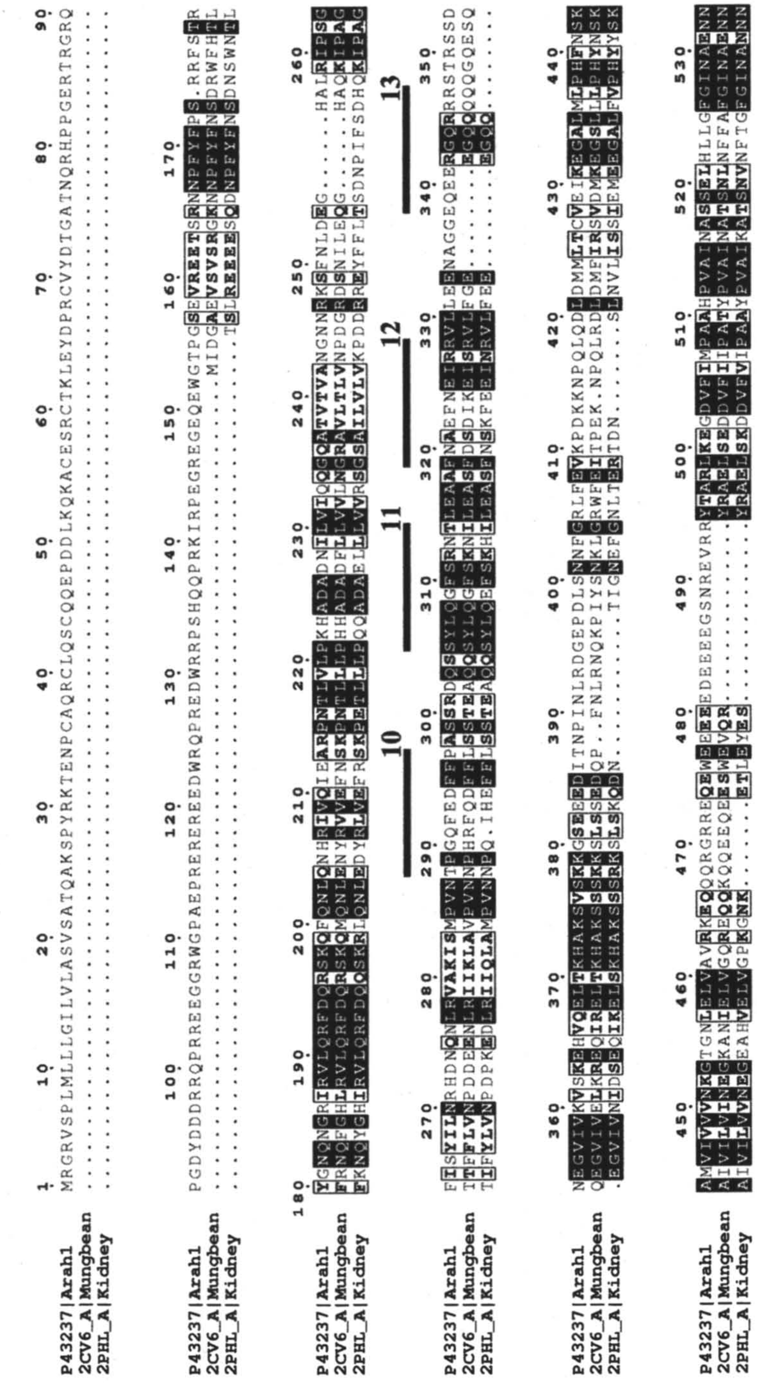
Annotation of the multiple sequence alignment with the location of the Ara h 1 allergenic epitopes 10 through 13 (*17*) is also shown on Figure 3 as black bars above the corresponding sequence of the epitope. For these four epitopes, we observe variable degrees of sequence identity/similarity. For example, epitopes 10 and 12 have 30 and 60% sequence identity/similarity, respectively. Epitope 11 has the highest sequence identity/similarity of 80%. Inspection of the alignment for epitope 13 reveals that there are only four identical/similar residues and that both mungbean and kidney bean have a significant portion of the epitope (between four and six residues) that is not present. When the multiple sequence alignment of these epitopes is further annotated by indicating which residues are required for IgE binding an interesting pattern emerges (Figure 4). For epitope 10, three of the four residues required for IgE binding are completely conserved with the fourth being conserved between Ara h 1 and mungbean. For epitope 11, again three of four residues for IgE binding are completely conserved; the fourth being conserved between Ara h 1 and mungbean. For epitope 12, one of two residues for IgE binding are conserved, and for epitope 13 the sole residue shown necessary for IgE binding is conserved for all three proteins.

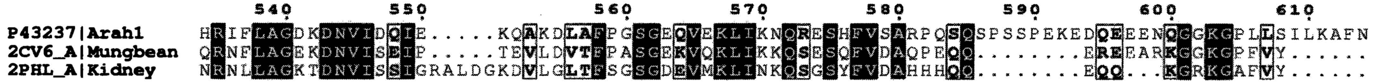
For Ara h 1 allergenic epitopes 10 and 11, the sequences are essentially identical with respect to their involvement in IgE binding. This leads to some interesting questions. If epitopes 10 and 11 are major contributing epitopes for Ara h 1 allergenicity, then why do we not see more pronounced allergenicity against mungbean and kidney bean in the human population, if there is a propensity for the IgE of peanut sensitive individuals to recognize these sequences? Also, if in the search for peanut cultivars that look more like mungbean or kidney bean in key allergenic regions, would our efforts be better spent looking at regions of greater dissimilarity? For example, while Ara h 1 epitope 12 does have one of two residues necessary for IgE binding conserved among the proteins in the alignment (a phenylalanine) the other residue is dissimilar (a phenylalanine for Ara h 1, and an isoleucine and a lysine for mungbean and kidney bean, respectively). If a peanut cultivar were found to have an amino acid besides phenylalanine at this position, would IgE binding still occur at this allergenic epitope? For Ara h 1 epitope 13, the sole amino acid involved in IgE binding is completely conserved. However, it appears that many residues for this epitope (between four and six) do not have structural counterparts in mungbean and kidney bean. Does epitope 13 reside in a position within the three-dimensional allergen significantly different from those of the other two proteins? Although we have only discussed sequence differences for these four epitopes, we can immediately see through our comparison of the Ara h 1 allergen and their less allergenic counterparts, epitopes 12 and 13 emerge as "regions of interest" for further structural comparison and analysis.

Ara h 1 Homology Model Generation, Structural Alignments and Annotation

Three homology models of Ara h 1 were built using SWISS-MODEL, 3D-JIGSAW and Phyre homology modeling servers. In each case, the X-ray crystal structure of the kidney bean phaseolin (PDB ID 2PHL) was selected as a template based on high sequence homology (*20*). As noted previously, the proteins from mungbean and kidney bean do not contain the 150 residue N-terminal leader sequence present for the Ara h 1 precursor protein (*56, 57*). Therefore this N-terminal leader sequence was removed from the Ara h 1 query sequence prior to submission to the homology modeling servers. Due to the high sequence conservation between the target (Ara h 1) and template sequences (kidney bean 2PHL), it is not surprising that the Ara h 1 homology model and the kidney bean phaseolin structure are essentially identical, and are remarkably similar to previously reported Ara h 1 homology models (*10, 17, 19*).

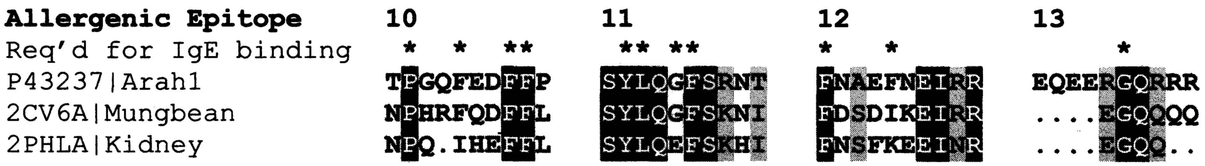
When the Ara h 1 homology models are superimposed onto the kidney bean X-ray structure, and the allergenic epitopes annotated according to amino acid identity/similarity and residues involved in IgE binding, we note differences among the three proteins that have implications for allergenicity. For example, when we again examine epitopes 10 and 11 (Figure 5) we see that these epitopes reside within solvent-inaccessible, hydrophobic regions involved in formation of the overall trimeric structure (for a review, see Maleki *et al.* (*10*)). As such, it is less likely that these epitopes will present their binding surfaces in a way necessary for efficient IgE binding if the allergens are presented to the immune system in an undigested form. One of our "regions of interest", Epitope 12, resides at an intermediate position being involved in hydrophobic protein-protein interactions and also presenting surfaces capable of efficient IgE binding. For our other region of interest, epitope 13, we see the greatest structural heterogeneity with respect to the three derived homology models and the template kidney bean structure. Here, the homology models posses either somewhat distended loops (for models returned from SWISS-MODEL (yellow) and Phyre (magenta)) or an alpha helix (for the model returned from 3D-JIGSAW (blue)). Based upon our multiple sequence alignment analysis and homology model generation and comparison, we see that epitope 13 may be structurally unique to Ara h 1, would reside in a surface-accessible region of the protein, and that there is no sequence/structural counterpart in the kidney bean template.


P43237|Arah1
2CV6_A|Mungbean
2PHL_A|Kidney
10
11
12
13



```
                    540       550             560       570       580       590       600       610
P43237|Arah1      HRIFLAGDKDNVIDQIE.....KQAKDLAFPGSGEQVEKLIKNQRESHFVSARPQSQSPSSPEKEDQEEENQGGKGPLLSILKAFN
2CV6_A|Mungbean   QRNFLAGEKDNVISEIP.....TEVLDVTFPASGEKVQKLIKKQSESQFVDAQPEQQ.........EREEARKGGKGPFVY......
2PHL_A|Kidney     NRNLLAGKTDNVISSIGRALDGKDVLGLTFSGSGDEVMKLINKQSGSYFVDAHHHQQ.........EQQ...KGRKGAFVY......
```

Figure 3. ClustalW sequence alignment of major peanut allergen Ara h 1 (top sequence, NCBI Accession P43237 (42)), mungbean (middle sequence, NCBI Accession 2CV6A (52)) and kidney bean phaseolin (bottom sequence, NCBI Accession 2PHLA (20)). All three belong to the vicilin subfamily of the cupin superfamily. Horizontal bars indicate positions of Ara h 1 epitopes 10 – 13 (17). All sequence alignments were rendered by use of ESPript (55).



Allergenic Epitope	**10**	**11**	**12**	**13**
Req'd for IgE binding	* * **	** **	* *	*
P43237|Arah1	TPGQFEDFFP	SYLQGFSRNT	FNAEFNEIRR	EQEERGQRRR
2CV6A|Mungbean	NPHRFQDFFL	SYLQGFSKNI	FDSDIKEIRR	....EGQQQQ
2PHLA|Kidney	NPQ.IHEFFL	SYLQEFSKHI	FNSFKEEINR	....EGQQ..

Figure 4. Regions of the clustalW multiple sequence alignment for Ara h 1, mungbean and kidney bean seed-storage proteins that correspond to Ara h 1 allergenic epitopes 10 – 13. Conserved residues are highlighted in black while those of the same approximate size and hydrophobicity are highlighted in gray. Residues shown necessary for serum IgE binding are indicated with an asterisk (17).

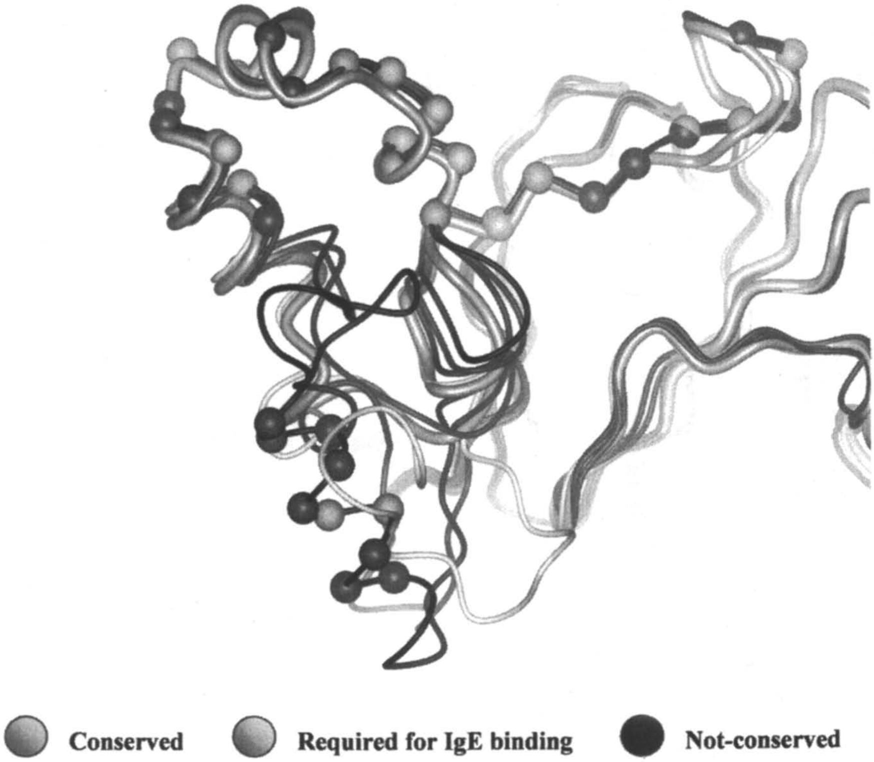


Figure 5. Backbone representation of Ara h 1 homology models generated from SWISS-MODEL (yellow), 3D-JIGSAW (blue) and Phyre (magenta) superposed upon the 2PHL X-ray crystal structure template from kidney bean (gray). Conserved residues are represented by green spheres located at corresponding backbone alpha-carbons. Those necessary for serum IgE binding are represented by cyan spheres. Nonconserved residues are represented by gray spheres. The location of IgE epitopes 10-13 are indicated (17). (See page 1 of color insert.)

Comparative Studies of Ara h 3

Initial Database Searching, Database Building

As with our studies of Ara h 1, our comparative studies of Ara h 3 (11S glycinin) begins with a search of available protein databases to find representative sequences. A keyword text search for "Ara h 3" of the NCBI database yields 11 protein entries, three of which are from peanut. Accessions "AAT39430" (Kang and Gallo-Meagher, unpublished direct submission to NCBI) and "AAC63045" (*16*) represent sequences derived from clones liberated from peanut cDNA libraries. Accession "AAM93157" represents a sequence corresponding to a cDNA clone of a trypsin inhibitor with homology to peanut allergens Ara h 3 and Ara h 4 (*13*). The recombinant protein corresponding to AAC63045 was expressed in *E. coli*, was further tested for serum IgE binding from patients with peanut sensitivity, and the location of allergenic epitopes mapped (*16*). Because this protein sequence was experimentally determined to bind serum IgE from peanut sensitive individuals, it was used for all subsequent Ara h 3 comparative analyses.

Sequence Comparison, Alignment, and Annotation

Submission of the AAC63045 Ara h 3 sequence to the NCBI BLAST server returned a distribution of over 300 BLAST hits on the query submitted December 2006. Of the most homologous sequences, there are three similar proteins that have structure entries in the Protein Data Bank; a soybean proglycinin (PDB entry 1FXZ (*21*) plus two point mutants of 1FXZ (1UD1, a C88S mutant, and 1CUX, a C12G mutant (*58*)). The sequence corresponding to 1FXZ was saved in FASTA format, a clustalW multiple sequence alignment generated, and the subsequent multiple alignment file was then uploaded to the ESPript server using a Risler Similarity Global Score of 0.7 (Figure 6).

Inspection of the multiple sequence alignment reveals that there is a high degree of identity between the sequences. The position of the post-translational NG cleavage site responsible for physical separation of the acidic (sequence to the right) and basic (sequence to the left) subunits for each protein are indicated with an "X" (*59-61*). Here 294 residues out of 507 (58%) are identical when compared to soybean (476 amino acids in length). Furthermore, 80 additional residues are of similar size and hydrophobicity to soybean. Therefore over 70% of the amino acids for the Ara h 3 protein are either identical or similar to those from the closely related soy counterpart. Also evident are two sequence insertions for Ara h 3 (13 and 28 amino acids respectively).

Annotation of the multiple sequence alignment with the location of the Ara h 3 allergenic epitopes 1 through 4 (*16*) is also shown on Figure 6 as black bars

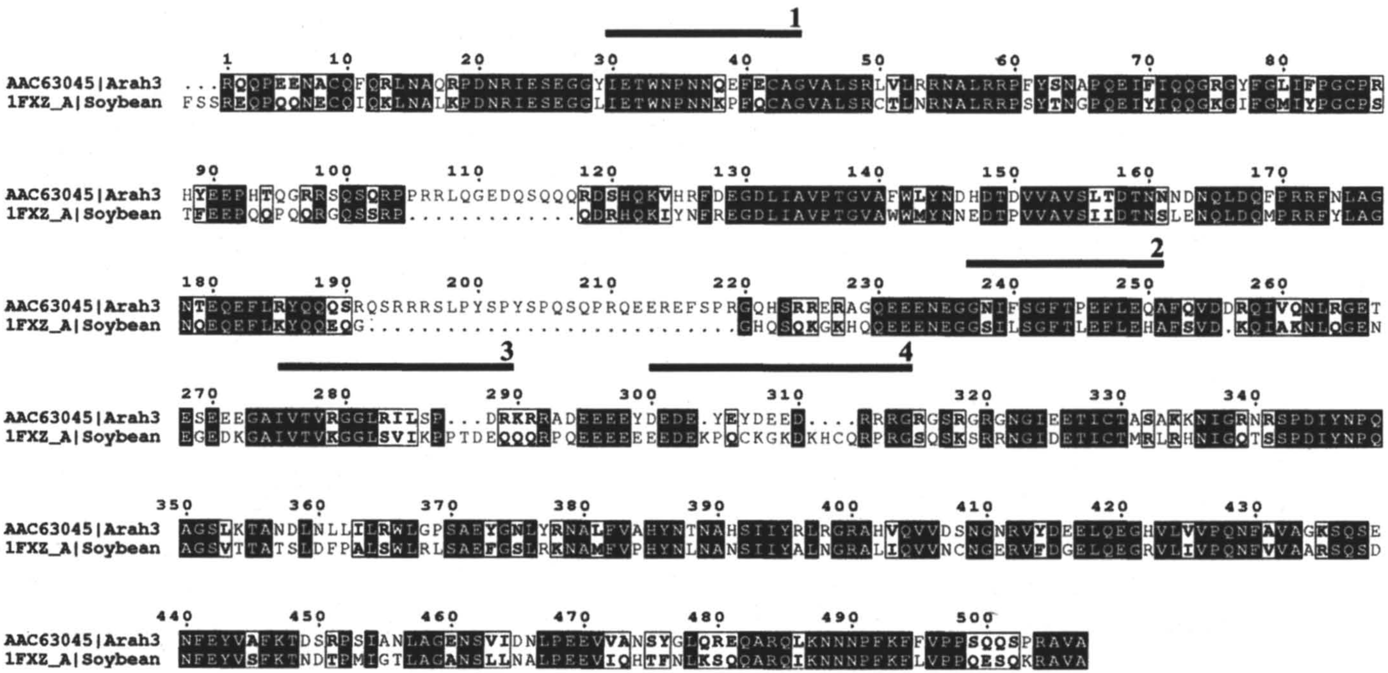


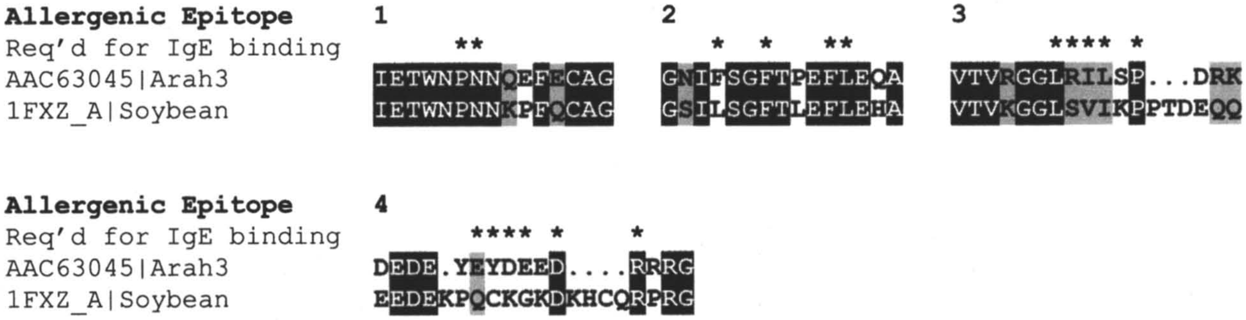
Figure 6. ClustalW sequence alignment of major peanut allergen Ara h 3 (top sequence, NCBI Accession AAC630045 (16)*), and soybean proglycinin (bottom sequence, NCBI Accession 1FXZA* (21)*). Both belong to the glycinin subfamily of the cupin superfamily. Horizontal bars indicate positions of Ara h 3 epitopes 1 – 4* (16)*. The location of a conserved post-translational Asn-Gly cleavage site that separates acidic and basic subunit chains is indicated above the Ara h 3 sequence by an "X"* (59-61)*.*

above the corresponding sequence of the epitope. For these four epitopes, we again observe variable degrees of sequence identity/similarity. Epitopes 1 and 2 show the greatest degree of similarity to corresponding sequences from the soybean sequence. Epitope 1 has 12 of 15 residues conserved plus an additional two that are similar (93%). Epitope 2 has 11 of 15 residues conserved plus an additional residue that is similar (80%). Epitope 3 has 7 of 15 residues conserved with an additional 5 that are similar (87%). Epitope 4 is the most dissimilar, having 7 of 15 residues conserved with only one additional similar amino acid (53%). Epitopes 3 and 4 also have a number of additional residues present in the corresponding soybean sequence that span the extent of these Ara h 3 epitopes (three and five residues respectively). When the multiple sequence alignment of these epitopes is further annotated by indicating which residues are required for IgE binding, we note several interesting patterns (Figure 7). For epitope 1, both residues required for IgE binding are completely conserved. For epitope 2 we see that three of four residues for IgE binding are conserved. For epitope 3, four residues necessary for IgE binding are similar, but only one is conserved. Finally, epitope 4 shows considerable dissimilarity, only having three of six conserved or similar residues necessary for IgE binding. Interestingly, the two C-terminal residues of Ara h 3 epitope 3 and all of the residues of the Ara h 3 epitope 4 reside in a region corresponding to the soybean glycinin "hypervariable region" residues P249 through T296 as described by Nielsen and co-workers (*62, 63*).

As with our comparative studies of Ara h 1, we see a number of sequence trends that may help us explain the differing allergenic profile for these two very similar proteins. We note that the sequence for epitope 1 is essentially identical to the corresponding sequence in soybean. For epitope 2, the sequences are highly homologous with the exception of one key residue necessary for IgE binding (a phenylalanine versus a leucine). For epitope 3, the sequences share a high degree of homology, but there are three residues that, while having the same approximate size and hydrophobicity, may impart differential and efficient IgE binding due to subtle chemical and space considerations. Epitope 4 shows significant heterogeneity with distinct per-residue differences for amino acids involved in IgE binding. Therefore, in our search for peanut varieties that "look" more like soybean in key Ara h 3 allergenic regions, our efforts may be better spent focusing on sequences that encompass epitopes 3 and 4.

Ara h 3 Homology Model Generation, Structural Alignments and Annotation

As we saw in our comparative studies of Ara h 1, the location of the allergenic epitopes may be of importance with respect to how accessible any one epitope is to serum IgE. Therefore, three homology models of Ara h 3 were built using SWISS-MODEL, 3D-JIGSAW and Phyre homology modeling servers. In



```
Allergenic Epitope     1                 2                 3
Req'd for IgE binding       **              *  *   **            **** *
AAC63045|Arah3         IETWNPNNQEFECAG   GNIFSGFTPEFLEQA   VTVRGGLRILSP...DRK
1FXZ_A|Soybean         IETWNPNNKPFQCAG   GSILSGFTLEFLEHA   VTVKGGLSVIKPPTDEQQ

Allergenic Epitope     4
Req'd for IgE binding        **** *    *
AAC63045|Arah3         DEDE.YEYDEED....RRRG
1FXZ_A|Soybean         EEDEKPQCKGKDKHCQRPRG
```

*Figure 7. Regions of the clustalW multiple sequence alignment for Ara h 3 soybean proglycinin seed-storage proteins that correspond to Ara h 3 allergenic epitopes 1- 4. Conserved residues are highlighted in black while those of the same approximate size and hydrophobicity are highlighted in gray. Residues shown necessary for serum IgE binding are indicated with an asterisk (*16*).*

each case, the X-ray crystal structure of the soybean proglycinin (PDB ID 1FXZ) was selected as a modeling template (*21*).

Examination of the published 1FXZ X-ray crystal structure reveals that sections of the protein are not present (Figure 8). In particular, structure coordinates are missing for residues F92 – D109, E179 – E193, N228 – D232, and the much larger segment corresponding to the soybean glycinin hypervariable region and including the post-translational NG cleavage site (P249 – T296) (missing regions indicated by pairs of similarly shaded spheres). Rotation of the protein by 90 degrees (Figure 8, right) reveals that a majority of the missing structure resides on one outer, lateral face. These deletions could be the result of proteolysis during protein purification, or due to polypeptide flexibility and an inability to resolve these regions due to insufficient electron density. It is important to note the absence of structure data for the homology template structures used because some computational servers will return homology models with these sections deleted as well (*e.g.*, Phyre).

In an effort to obtain a more complete template structure, the missing sections of the 1FXZ X-ray structure were modeled by submission of the complete 1FXZ amino acid sequence to the SWISS-MODEL homology modeling server. The resultant complete model soybean glycinin structure overlaid on the 1FXZ template structure is shown in Figure 9A. We note that missing sections for F92 – D109 and E179 – E193 model as regular elements of secondary structure (alpha helices) and that the shorter section for N228 – D232 modeled as a loop. What is surprising is the apparent lack of predicted secondary structure for the much larger hypervariable segment missing between P249 and T296. As noted previously, homology modeling algorithms are good at fold recognition, but can have difficulties predicting detailed secondary structure elements for lengthy sequences without a known structural template. It may be possible that this region of the soybean glycinin does not contain any elements of structure and may be classified as being intrinsically disordered or dynamic. This region also represents the glutamate-rich hypervariable C-terminus of the acidic subunit, and the location of the post-translational cleavage site which physically separates acidic and basic subunits for Ara h 3 and the soybean glycinin. Thus polypeptide-chain dynamics may be particularly relevant. To better determine the possibility that this particular region of "structural uncertainty" may contain regular elements of secondary structure, we performed additional secondary structure prediction, the results of which are discussed later in the Chapter.

Raw, unrefined homology models for the complete Ara h 3 sequence were returned from the SWISS-MODEL and 3D-JIGSAW homology modeling servers, while the homology model returned from Phyre lacked the sections missing from the 1FXZ template structure. Superposition of all of the homology models onto the 1FXZ template structure showed excellent structural correlation for those regions of the proteins that had structural counterparts in the soybean

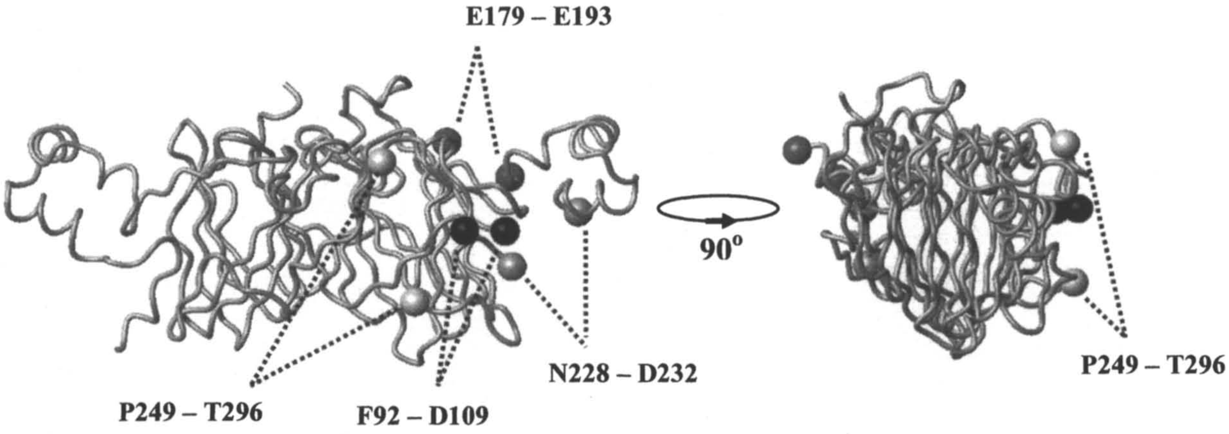

E179 – E193
90°
N228 – D232
P249 – T296
F92 – D109
P249 – T296

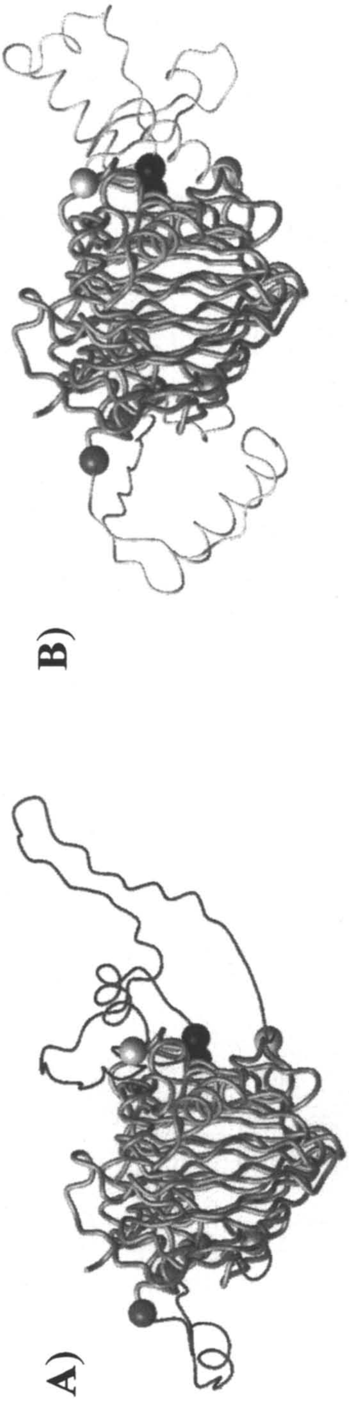
Figure 8. Backbone representation of one monomer from the soybean proglycinin X-ray crystal structure (PDB ID 1FXZ (21)). Spheres of similar shade indicate where sections of the structure are missing due to insufficient electron density and/or proteolysis.

template structure (data not shown). In Figure 9B, we show only the SWISS-MODEL model (yellow) superimposed onto the 1FXZ soybean X-ray crystal structure (the 3D-JIGSAW and Phyre homology models are not shown for clarity). Even in its unrefined state, regular secondary structure elements are predicted for those regions of the Ara h 3 allergen that lack a structural counterpart in the soybean template (four alpha helices). While the exact articulation/position of the these helices would be expected to differ from the Ara h 3 protein as it actually exists *in vitro* or *in vivo*, it is striking that where we see regular secondary structure predicted for the Ara h 3 molecule, there is structural uncertainty predicted for corresponding regions of the complete soybean glycinin molecule (Figure 9C).

Figure 9D represents an overlay of the Ara h 3 SWISS-MODEL homology structure (yellow) and the complete soybean glycinin model structure (red) onto the 1FXZ template structure (gray). Furthermore the location of the Ara h 3 allergenic epitopes have been highlighted as thicker backbone representations colored in magenta for epitopes 1 through 3 and yellow for epitope 4. We note that Ara h 3 epitopes 1 – 3 reside in regions of structural certainty, lining up with known regions of the 1FXZ template structure. For Ara h 3 epitope 4, we see that this epitope resides on a lateral face that shows the greatest degree of structural uncertainty with respect to the soybean model. In our analysis of the sequence similarity of Ara h 3 epitopes with corresponding residues of the soybean glycinin protein, we saw that epitope 4 was the most dissimilar. Does the fact that for our ensemble of Ara h 3 homology models epitope 4 resides in a location that may display elements of structural uncertainty or potential polypeptide-chain dynamics help explain their differential allergencity? Are there elements of polypeptide-chain dynamics present on the lateral face of the soybean protein that somehow preclude IgE recognition to the similar epitope sequences for the soybean glycinin? Despite the need for further experimental investigation to answer these questions, we are nonetheless able to formulate hypotheses regarding differential Ara h 3 and soybean glycinin allergenicty that can be directly tested in the wet lab.

Secondary Structure Prediction for the Potentially Disordered Section of Soybean Proglycinin, Comparison to Ara h 3

We noted in the previous section that there are several sequence segments missing from the 1FXZ soybean glycinin X-ray crystal structure that was used as the homology modeling template for generation of our ensemble of Ara h 3 homology models. Therefore, we submitted the sequence corresponding to the complete 1FXZ soybean glycinin protein to the SWISS-MODEL homology modeling server. For our returned model of the complete glycinin structure (Figure 9A), two of the four stretches were predicted to be helical, and a shorter


A)
B)

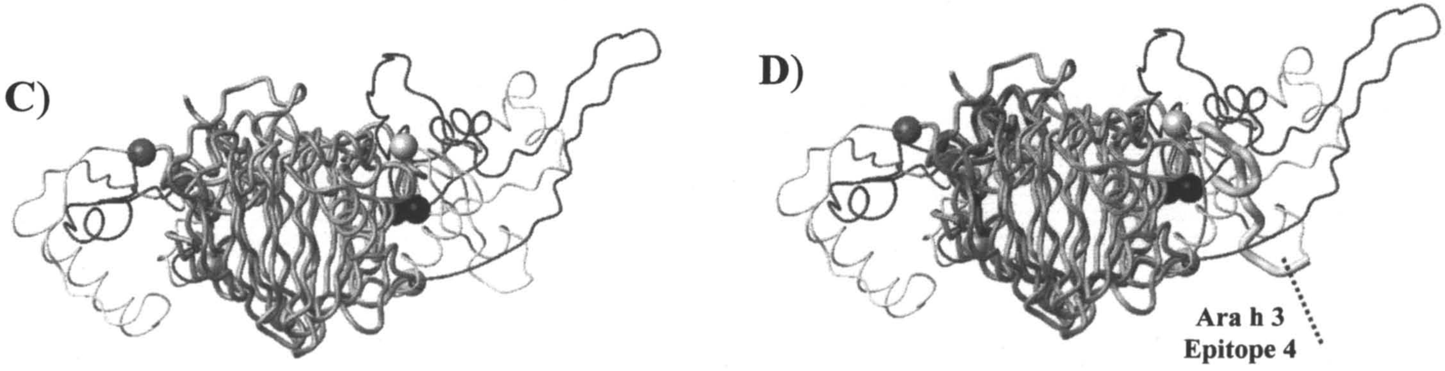


Figure 9. A) Superposition of the SWISS-MODEL soybean proglycinin model structure (red) and the 1FXZ X-ray soybean proglycinin structure template (21). Submission of the complete soybean proglycinin amino acid sequence to the SWISS-MODEL homology modeling server was done in an effort to obtain a more complete structure for comparison to homology models generated for Ara h 3. B) Ara h 3 homology model generated from SWISS-MODEL (yellow) superimposed upon the 1FXZ X-ray soybean proglycinin crystal structure template (gray). C) Superposition of the Ara h 3 homology model generated from SWISS-MODEL (yellow), the SWISS-MODEL soybean proglycinin model structure (red) and the 1FXZ X-ray crystal structure template (gray). D) Annotation of the overlaid SWISS-MODEL structures with the location of Ara h 3 allergenic epitopes 1-4 (16). The relatively sequence-similar epitopes 1-3 are highlighted as thicker backbone magenta representations while the sequence-dissimilar epitope 4 is highlighted in yellow.
(See page 2 of color insert.)

stretch predicted as a loop joining segments of regular secondary structure. The longest stretch (the C-terminal 43 amino acid portion of the soybean glycinin acidic subunit, and encompassing the corresponding residues for Ara h 3 epitope 4) failed to coalesce (predicted) into any recognizable secondary structure, and may represent a region of structural uncertainty or may be intrinsically disordered. Because this Ara h 3 allergenic epitope was shown to be most dissimilar as compared to the sequence corresponding to soybean, we hypothesized that this could help explain the differential allergenic profile of the two proteins. We wished to further characterize the possible propensity for this region not to adopt regular elements of secondary structure and could in fact be intrinsically disordered. For this, we submitted the 43-amino acid soybean sequence, and the corresponding 35-amino acid Ara h 3 sequence to several available secondary prediction servers including DISOPRED (*64*), PsiPred (*65*), SEG (*66, 67*) and SAM-T99 (*68, 69*), the results of which are shown in Figure 10. For soybean, DISOPRED analysis predicts that this region is completely disordered. SEG analysis predicts a preponderance of the protein to be of low complexity (46%), and both PsiPred and SAM-T99 predict essentially no secondary structure for this sequence with the exception of a potential 3-turn helix at the N-terminus.

The results for the 35-amino acid Ara h 3 sequence are shown at the bottom of Figure 10, and aligned with the corresponding sequence from soybean. Here we see slightly different results. As with the section from soybean, DISOPRED predicts the peptide to be completely disordered. However, PsiPred and SAM-T99 predict elements of regular secondary structure (helices) for this region. Interestingly, both PsiPred and SAM-T99 predict a helix for the first section of the Ara h 3 peptide, but essentially no helical content for the N-terminal section of the soybean peptide, despite the high sequence homology/similarity for residues that span the predicted Ara h 3 helix. Closer examination reveals that there are seven sequential glutamate residues in the soybean sequence and four sequential glutamate residues for the Ara h 3 sequence. The additional glutamate residues for the soybean protein may preclude folding of this region of the protein into a regular secondary structural element such as a helix due to electrostatic repulsion of the negatively charged sidechains. These analyses serve to corroborate our previous results for our homology models returned from SWISS-MODEL and 3D-JIGSAW. Therefore, it is possible that there is a degree of intrinsic disorder associated with this region of the soybean glycinin where there is predicted structure present for Ara h 3.

Ab initio Structure Prediction and Molecular Dynamic Simulations of the Ara h 1 N-terminal Leader

In our initial analysis of the clustalW multiple sequence alignment for Ara h 1 (P43237) (*42*), mungbean (2CV6A) (*52*) and kidney bean (2PHLA) (*20*)

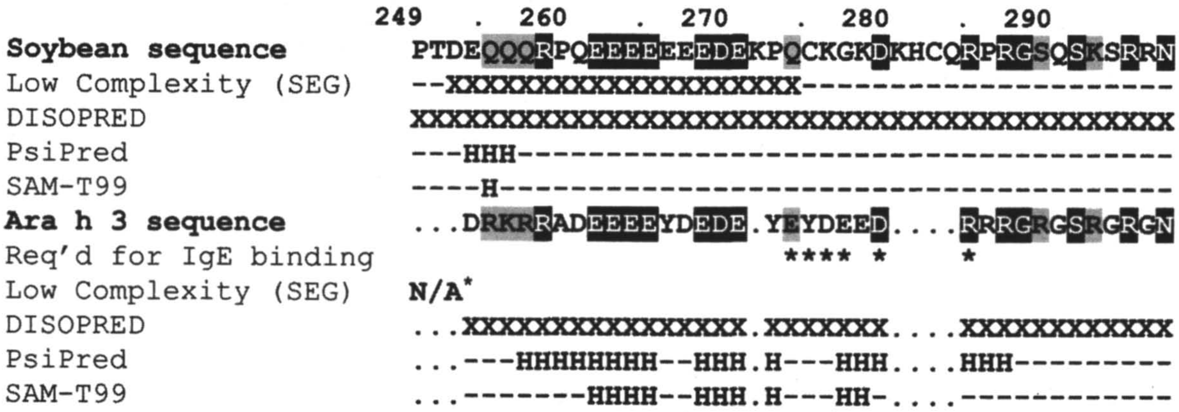

249 . 260 . 270 . 280 . 290
Soybean sequence PTDEQQQRPQEEEEEEEDEKPQCKGKDKHCQRPRGSQSKSRRN
Low Complexity (SEG) --XXXXXXXXXXXXXXXXXXXX---------------------
DISOPRED XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX
PsiPred ---HHH-------------------------------------
SAM-T99 ----H--------------------------------------
Ara h 3 sequence ...DRKRRADEEEEYDEDE.YEYDEED....RRRGRGSRGRGN
Req'd for IgE binding ***** * *
Low Complexity (SEG) N/A*
DISOPRED ...XXXXXXXXXXXXXXXX.XXXXXXX....XXXXXXXXXXXX
PsiPred ...---HHHHHHHH--HHH.H---HHH....HHH---------
SAM-T99 ...-------HHHH--HHH.H---HH-....------------

*Figure 10. Secondary structure prediction for the potential disordered section (residues P249-N291) and the corresponding sequence from Ara h 3. Conserved residues are highlighted in black; those with the same approximate size and hydrophobicity are highlighted in gray. For the Ara h 3 sequence, residues shown necessary for IgE binding are indicated with asterisks (*16*). *At the time of manuscript preparation, SEG secondary Ara h 3 structure prediction results were not available.*

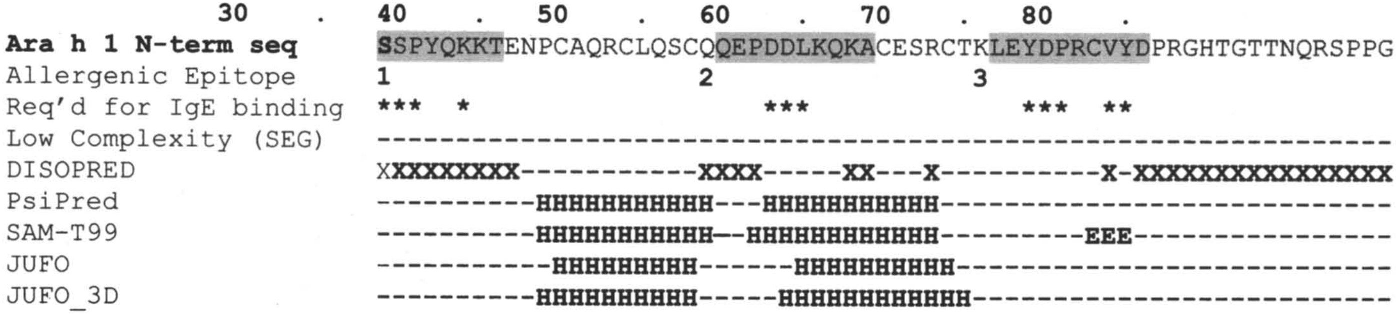
proteins, we noted that Ara h 1 possesses a N-terminal leader sequence of approximately 150 amino acids not present for the other two proteins. As first described by De Jong (*56*), and later by Wichers and co-workers (*57*), this N-terminal section is cleaved off *in planta* from the mature Ara h 1 protein. While physically separated from the mature Ara h 1 allergen, this N-terminal leader sequence is still present in peanut and contains three of the 21 reported allergenic epitopes (*17*). Furthermore, this N-terminal leader sequence was shown to be similar to a class of antifungal proteins (*70*) and hypothesized to possess a β-α-β molecular motif possibly similar to the cysteine-rich AH-AMP1 antimicrobial protein from radish (*71*).

Submission of the 150 residue Ara h 1 N-terminal leader sequence to the NCBI BLAST server resulted in 41 hits. However, there were no experimental structural data available for any of them. Therefore, we wished to employ available secondary structure prediction and subsequent *ab initio* three-dimensional structure prediction resources to derive a hypothetical structure of this sequence. Examination of the Ara h 1 UniProt entry (P43237) indicates that the first 25 amino acids of the N-terminal leader sequence may be a signal peptide. Therefore, these 25 amino acids were removed from the sequence in subsequent analyses.

Analysis of this section using DISOPRED, PsiPred, SEG, SAM-T99, JUFO (*72*) and JUFO_3D (*73*) results in two possible structural domains (residues S26 through G76 and P115 through Q140 respectively). Because the first of these two possible structural domains contains the first three of the 21 Ara h 1 allergenic epitopes, we have presented results for this section only (Figure 11). Here we see a prediction consensus of two closely spaced helices. When further annotated with the location of the first three Ara h 1 epitopes (sequence shaded in gray) and the position of residues necessary for serum IgE binding, we see the potential for both linear and conformational epitopes.

We then extended our analyses to predict a complete three-dimensional fold using the Robetta protein structure prediction resource available through the University of Washington (http://robetta.bakerlab.org) (*74-77*). Submission of the sequence S26 through G76 to the Robetta server using the default submission parameters returned a very plausible helix-turn-helix model structure flanked by segments of unfolded protein. Interestingly, all six cysteine residues of the model structure reside within the core of the protein fold, and are within close proximity giving rise to the possibility of several disulfide linkages.

We employed the program "SSBOND" developed by Hazes and co-workers to determine the potential for disulfide bond formation (*78*) within the core of the model. Submission of the model coordinates resulted in six different combinations of cysteine pairing (C37-C61, C37-C70, C41-C57, C41-C61, C45-C57, C61-C70). We initially noted that C45 could only pair with C57. This would result in C41 only being able to pair with C61, and subsequently C37 only being able to pair with C70. Therefore, of the six possible disulfide



```
             30    .   40    .   50    .   60    .   70    .   80    .
Ara h 1 N-term seq     SSPYQKKTENPCAQRCLQSCQQEPDDLKQKACESRCTKLEYDPRCVYDPRGHTGTTNQRSPPG
Allergenic Epitope     1                   2                3
Req'd for IgE binding  ***  *                  ***             ***  **
Low Complexity (SEG)   ---------------------------------------------------------------
DISOPRED               XXXXXXXXX-----------XXXX-----XX---X----------X-XXXXXXXXXXXXXXXX
PsiPred                ----------HHHHHHHHHHH---HHHHHHHHHHH----------------------------
SAM-T99                ----------HHHHHHHHHHH--HHHHHHHHHHHH---------EEE----------------
JUFO                   -----------HHHHHHHHH------HHHHHHHHHH---------------------------
JUFO_3D                ----------HHHHHHHHHH-----HHHHHHHHHHHH--------------------------
```

Figure 11. Results of secondary structure prediction for residues S27-E89 of the Ara h 1 N-terminal leader sequence. The location of Ara h 1 allergenic epitopes 1-3 are highlighted in gray. Residues shown necessary for IgE binding are indicated with asterisks (17).

combinations, there is only one in which all six cysteine residues can be involved in disulfide bond formation. The locations of the disulfide bridges within the core of the protein fold suggests that this possible Ara h 1 globular domain would be highly resistant to thermal denaturation.

Using our Robetta model structure, we created disulfide bonds between C37 and C70, C41 and C61, and C45 and C57, and refined the structure using the AMBER8 suite of molecular dynamics algorithms (*79*). We have termed this structure "Arah1GD1" (Ara h 1 Globular Domain 1), and a minimized and annotated representation is presented in Figure 12. We further subjected the model protein to nanosecond solvated molecular dynamics simulations where we incrementally increased the temperature from 300 to 500 K, and noted the propensity for this disulfide-rich protein to maintain its overall structure throughout the simulation trajectory. While beyond the scope of what is presented here, a complete and detailed manuscript describing the details, results and analyses of the molecular dynamic simulations of the model structure is in preparation and will be published elsewhere.

Further annotation of the disulfide-rich model structure with the location of potential pepsin cleavage sites at pH 1.3 (using the PeptideCuter analysis tool available through the Expert Protein Analysis System Proteomics server available through the Swiss Institute of Bioinformatics, http://au.expasy.org/tools/peptidecutter) (*80*), suggests that even upon treatment with the pepsin protease, the domain would remain largely intact and retain all three allergenic epitopes. Therefore, while at this time our structure of this Ara h 1 N-terminal fragment is purely hypothetical, we see the potential for an Ara h 1 allergen that is highly resistant to thermal denaturation and proteolytic cleavage. For studies concerning the acute allergenicity of peanut, and the apparent ability of peanut allergens to retain their allergenic profile despite various processing regimes (*e.g.* roasting/digestion), these results may be of particular relevance.

Conclusions

The use of bioinformatic tools, resources, and strategies to study food allergens such as those in peanut can provide valuable information regarding the variable degrees of allergenicity of food allergens when compared to their less allergenic counterparts. However, the study of allergen structure using computational approaches, while exciting and increasingly successful, can be complex. Here we demonstrate a general series of analysis steps and procedures as applied to comparative structural studies of peanut allergens Ara h 1 and Ara h 3 using freely available bioinformatic resources.

Through our comparison of Ara h 1 to similar seed-storage proteins from kidney bean and mungbean we are able to characterize subtle structural differences between the proteins that may help explain their differential

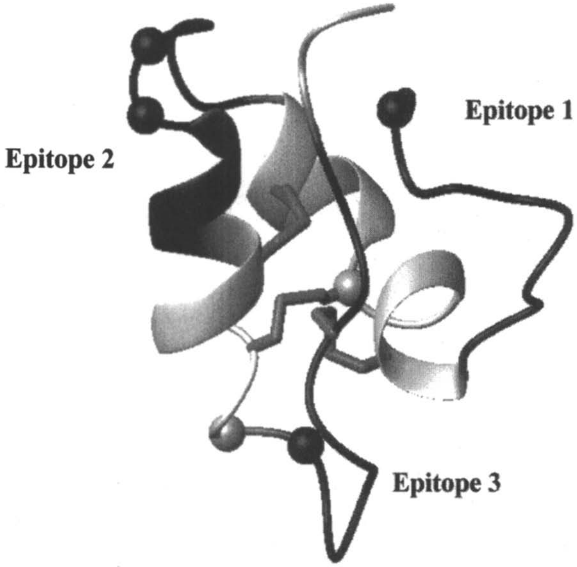


*Figure 12. Hypothetical structure of Arah1GD1 (Ara h 1 Globular Domain 1). Ara h 1 allergenic epitopes 1-3 (*17*) are colored black. The locations of potential pepsin cleavage sites (pH 1.3) are indicated by black spheres (*80*). Potential stabilizing disulfide bonds are indicated.*

allergenic profile. While we have considered only four of the 21 allergenic epitopes of Ara h 1, we note that Ara h 1 epitopes 12 and 13 emerge as regions of interest for further experimental work. The amino acid sequence for Ara h 1 epitope 12 is highly homologous to sequences from kidney bean and mungbean. However, the presence of one non-conserved phenylalanine in the Ara h 1 sequence for this epitope appears to impart differential and significant allergenicity relative to kidney bean and mungbean. Ara h 1 epitope 13 is substantively dissimilar to corresponding sequences from kidney bean and mungbean. Based upon our ensemble of Ara h 1 homology models, and superposition of the models onto the X-ray crystal structure from kidney bean, we see that Ara h 1 epitope 13 resides in a solvent- and IgE-accessible location that may be structurally unique to Ara h 1.

Through our comparison of Ara h 3 to the similar protein from soybean, we see that the amino acid sequence for Ara h 3 epitope 1 is essentially identical to the corresponding sequence from soybean. For Ara h 3 epitope 2, the sequences are highly homologous with the exception of one key phenylalanine implicated in serum IgE binding. Epitope 3, while also highly homologous, contains three amino acids critical for IgE binding that are similar in size and hydrophobicity to the corresponding sequence from soybean, but due to subtle chemical and space considerations appears to impart differential allergenicity relevant to this particular epitope. The amino acid sequence for Ara h 3 epitope 4 is the most dissimilar relative to soybean, residing on one lateral face of the Ara h 3 homology model protein that is predicted to contain regular elements of secondary structure. However, the corresponding region in soybean may be intrinsically disordered or dynamic and may have structural consequences that help explain differential allergencity of Ara h 3 versus soybean glycinin. As with Ara h 1, we are able to identify Ara h 3 regions of interest for subsequent experimental follow up.

Finally, we used secondary structure prediction algorithms, *ab initio* structure calculation resources, and molecular dynamics simulations to generate and characterize a hypothetical structural model of "Arah1GD1", a possible globular domain of the cleaved-off N-terminal peptide of Ara h 1 that possesses three of the 21 allergenic Ara h 1 epitopes. In our model we noted the possibility of formation of three disulfide bridges within the core of the protein that would significantly stabilize the allergen against thermal denaturation. Furthermore, mapping of the locations of pepsin cleavage sites onto the model structure demonstrates that the allergenic epitopes would remain intact following proteolysis and digestion.

These comparative structural studies of peanut allergens Ara h 1 and Ara h 3 demonstrate the utility of bioinformatic tools, resources, and strategies to better understand the differential allergenicity of peanut allergens with their closely related, yet less allergenic, counterparts from other legume species. We also provide for a general series of steps that can be applied to the study of other food allergens. More importantly, these studies provide for hypotheses regarding allergen structure that can be tested experimentally. Here we identify key allergenic regions of Ara h 1 and Ara h 3 that can be structurally modified using recombinant techniques and subsequently tested for serum IgE binding, and suggest hypothetical allergen structure(s) resistant to thermal denaturation and proteolytic cleavage. Our analyses provide a foundation from which to find peanut cultivars that have reduced allergenicity, and assist in efforts to formulate specific "vaccines" from recombinant peanut sources as a means to improve the nation's nutrition and health and reduce the severity of peanut allergy.

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Chapter 21

An Integrated Concept for the Assessment of Unintended Immune Responses in Modern Food Biotechnology

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CODEX guidelines have been established for the risk assessment of GM food. The evaluation of allergenicity includes aspects of the source of the transferred gene, unintended effects of the transformation event, stability testing, bioinformatic tools for analysis of sequence homologies with identified allergens, and the use of sera from allergic patients. Discussions of the present concept address its reliability, especially the methods for a comparison with allergen sequences, and the potential need of additional tests. A WHO report on modern food biotechnology additionally asks for a holistic, integrative concept which includes the analysis of potential indirect consequences from the environment. New developments of functional foods, such as probiotics, combating infections, cell mediated or even autoimmune responses, will soon require the establishment of internationally harmonized risk assessment concepts in these areas.

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Besides foodborne infections, unintended immune responses represent a major hazard in food production. The understanding of the interaction between foods, nutrition, the development of the immune system, and immune responses to foods are an important element in the assessment of adverse reactions to foods.

The main strategy for avoiding adverse allergic reactions to foods among individuals sensitized to those foods has been the avoidance of the incriminated food. However, epidemiological studies fail to show simple associations, *e.g.* between maternal consumption of allergen during pregnancy and lactation and the development of food allergies (*1*). A low reported prevalence of peanut allergy has been observed in African and Asian countries despite high levels of environmental exposure to peanut. One possible explanation is that oral tolerance induction through early infant feeding protects these populations against allergic sensitization; there is growing understanding that the development and maintenance of immunological tolerance are lifelong processes which start already prenatally through changes of specific T cell responses (*1*).

With the development of modern food biotechnology and the use of genes and constituents from sources with no history of safe food use, the assessment of unintended immune responses to foods became even more important. Global trading and upcoming international regulations for risk assessment, risk management, and approval of foods produced with modern methods of biotechnology have resulted in the need of internationally harmonized concepts for the evaluation of immune responses. Whereas potential adverse immune responses induced by foods containing genetically modified organisms (GMO) have been the subject of intensive research and assessment by national authorities, many foods produced by more traditional methods have entered the markets without such a specified risk assessment according to international concepts.

International Regulations for Foods Derived from Modern Biotechnology

In response to the increased delivery of genetically modified (GM) foods to international markets, the Ad Hoc Intergovernmental Task Force on Food Derived from Biotechnology of the Codex Alimentarius Commission (Rome) agreed on principles for the human health risk analysis of GM foods. These principles dictate a case-by-case premarket assessment that includes an evaluation of both direct and unintended effects. They state that safety assessment of GM foods needs to investigate:

- direct health effects (toxicity),

- tendency to provoke allergic reactions (allergenicity),
- specific components thought to have nutritional or toxic properties,
- the stability of the inserted gene,
- nutritional effects associated with genetic modification and
- any unintended effects that could result from the gene insertion.

Of particular note, the task force broadened risk assessment to encompass not only health-related effects of the food itself, but also indirect effects of food on human health, *e.g.*, potential health risks derived from outcrossing (*2*). CODEX principles do not have a binding effect on national legislation, but are referred to specifically in the Agreement on the Application of Sanitary and Phytosanitary Measures of the World Trade Organization (SPS Agreement, see WTO 1995), and are often used as a reference in the case of trade disputes. In addition, the Cartagena Protocol of Biosafety (CPB) is a legally binding international instrument that regulates the transboundary movement of living modified organisms (LMOs) resulting from modern biotechnology with the objective of protecting the environment. The backbone of the protocol is the advance informed agreement requiring consent prior to the shipment and introduction of an LMO into the environment of an importing country.

The CODEX guidelines include a concept for an assessment of potential allergenicity of GM foods, including a decision tree elaborated in consultations of FAO/WHO. Risk-assessment protocols for food allergy examine four elements:

1. Allergenicity assessment (Is the food or elements in the food a potential allergen?)
2. Dose response assessment (Is there a safe concentration of the allergen?)
3. Exposure assessment (How likely is it that people will encounter the allergen)
4. Susceptible subpopulations (How do individuals prone to allergy react to this new food?)

Elements of an allergenicity assessment include a comparison of the sequence of the transferred gene (including the flanking regions at the site of insertion) with sequence motifs of allergenic proteins from databanks, an evaluation of the stability of the newly expressed proteins against digestion, and animal and immune tests, as appropriate (*3*).

Also the guidance document of the scientific panel on genetically modified organisms of the European Food Safety Agency (EFSA) for the risk assessment of genetically modified plants and derived food and feed (*4*) only considers allergenicity in the context of unintended immune responses and widely follows CODEX principles. This document additionally explains that allergenicity is not an intrinsic and fully predictable property of a given protein but is a biological activity requiring an interaction with individuals with a pre-disposed genetic background. Allergenicity therefore depends upon the genetic diversity and variability in atopic humans. CODEX and EFSA guidance documents mainly focus on the assessment of allergic immune responses and only briefly address other types of immune responses.

The Assessment of Indirect Effects from the Environment

Evidence for substantial environmental influences on health and food safety comes from work with environmental health indicators which show that agro-environmental practices have direct and indirect effects on human health, concluding that "the quality of the environment influences the quality and safety of foods" (*5*). Recently, a WHO report on modern food biotechnology pointed out the need for a holistic, integrated assessment of GM foods including environmental and socio-economic aspects (*6*). Therefore, evaluating the link between the environment, food production, and immune responses to foods was suggested to be an important aspect in the assessment of new foods as experiences from conventional methods of food production suggest that changes of immune responses within certain groups of consumers could be mediated by changes in the food production methods (*7*). Pollen-allergic individuals frequently present allergic symptoms after ingestion of several kinds of plant-derived food components presumably by cross-reactive structures. Unexpected immune responses to a naturally non-toxic protein transferred from beans to peas were blamed to be responsible for allergic lung damage observed in mice (*8*), possibly because of subtle structural changes when the protein was expressed in peas. Allergenic structures that sensitize pollen-allergic patients are also present in grass and weed pollen (*9*). Allergic potential of grasses and weeds, including sensitization and possibly also induction of tolerance, are well known. For example rice plants contribute a huge pollen load in agricultural fields during flowering which results in a seasonal trigger of hay fever and respiratory allergy in field workers and people living in the vicinity (*10, 11*). On the other hand, farmers who have grown up on farms present a lower prevalence of atopy (*12*). Recently detailed analysis of allergen-dependent switching patterns *in vivo* confirm that the farming environment protects against IgE responses of human B-cells from allergen-dependent, T_H2 cell mediated isotype switching. The protective effects of farm exposure were confined to T_H2-dependent IgG1, IgG4, and IgE expression and were allergen and switch stage specific. This suggests that distinct mechanisms regulate individual steps within allergen-induced class switching *in vivo* (*13*).

These results go in line with the hygiene hypothesis which suggests that limited exposure to bacterial and viral pathogens during early childhood results in an insufficient stimulation of T_H1 cells, which in turn cannot counterbalance the expansion of T_H2 cells, resulting in a predisposition to allergy. Exposure to some pathogens may reduce the risk of atopy by more than 60% (*14*).

Whereas the role of T_H2 cells producing interleukin IL4, IL-5, and IL-13 in allergic reactions is well-defined. New evidence describing a distinct proinflammatory T cell lineage called T_H17 cells, producing IL-17A, a cytokine that induces IL-8 and recruits neutrophils, may bring new aspects to the understanding of certain immune reactions to foods (*15*, *16*).

Unintended Immune Responses to Microorganisms in Foods

In the area of microorganisms and modern food biotechnology, the CODEX guidelines give advice for the conduction of food safety assessment of GM foods (*17*). In addition to the general CODEX assessment scheme for allergenicity, this document describes aspects of genetically modified microorganisms and mentions that a variety of microorganisms used in food production have a long history of safe use that predates scientific assessment. Few microorganisms have been characterized scientifically regarding the potential risks associated with the food they are used to produce, including, in some instances, the consumption of viable microorganisms. Furthermore, the CODEX principles of risk analysis are primarily intended to apply to discrete chemical entities such as food additives and pesticide residues, or specific chemical or microbial contaminants that have identifiable hazards and risks. Recombinant-DNA microorganisms that remain viable in foods may interact with the immune system in the gastrointestinal tract. Closer examination of these interactions will depend on the types of differences between the recombinant-DNA microorganism and its conventional counterpart; there is a need an assessment of viability and residence of microorganisms in the human gastrointestinal tract.

Also the guidance document of the scientific panel of the European Food Safety Authority (EFSA) on genetically modified organisms for the risk assessment of genetically modified microorganisms and their derived products intended for food and feed use (18) only remarks that particular attention should be paid to potential interactions with the gut microbiota and the evaluation of any effect on the digestive physiology and immune response of the host.

Internationally harmonized concepts for the safety assessment of complex immune responses induced by novel foods containing microorganisms are urgently desired as functional or dietetic food concepts are developing rapidly. There is growing appreciation for the role of the enteric flora in health and disease. Evidence for the role of commensal gut bacteria in the inflammatory bowel diseases, Crohn's disease, and ulcerative colitis have accumulated (19). Probiotics are established in the maintenance therapy for all these diseases. Most approaches for these diseases currently aim at modulating the immune response. A different concept is to consider probiotic therapy in terms of specific molecules modulating defined targets in the gut mucosa.

Western infants have delayed acquisition of several gut microbes and a reduced turnover of strains in the colon, indicating a low exposure to a small variety of environmental bacteria. An immunoflora study has examined how early intestinal colonization affects allergy in Swedish infants. In a meta-analysis of the effect of oral administration of probiotic bacteria on acute infectious diarrhea in children, the use of probiotics was associated with significantly reduced risks of diarrhoea. In addition, antibiotic treatment of respiratory infections could be reduced (*19*). Data also suggest that probiotics are more effective in preventing relapse of inflammatory process than in suppressing active disease (*19*).

Following these concepts we soon may also see improved bacteria as functional foods or therapies delivering anti-inflammatory cytokines or other biologically active molecules to the gut .

Individual Aspects of Unintended Immune Responses

In addition to environmental factors, the genetic disposition of individuals has often been mentioned as a major determinant of unintended immune reactions to foods. Many polymorphisms in molecules involved in the generation of immune responses have been described previously. Nutritional genomic studies have effectively demonstrated the consequences of genetic disposition in the different individual reactions to food ingredients and many single nucleotide polymorphism (SNP) studies have been reported in association with atopy, or allergic diseases. The impact of SNPs in cytokine-related genes on the severity of food allergy and atopic eczema in children has been described (*20*). Therefore, individual characteristics may need to be an additional aspect in the risk assessment of unintended immune reactions to foods in the future.

Criticisms and Suggestions for Improvements

Despite or in response to internationally elaborated concepts, there are continous discussions about these concepts (*21, 22*) and the possibility that the allergenic potential of GMOs may be increased due to the introduction of potential foreign allergens, to potentially upregulated expression of allergenic components caused by the modification of the wild type organism, or to different means of exposure. Based on the experience with isoforms of allergens with only slight modification of sequences but significant changes in allergenicity (*23*) the decision tree endorsed by CODEX may not exclude the allergenicity of a given GMO with certainty. An alternative improvement of the current evaluation was proposed; an experimental comparison of the wild-type organism with the whole GMO regarding their potential to elicit reactions in allergic individuals and to induce *de novo* sensitizations. Furthermore, the suggested assessment procedures should also be applied to natural cultivars in order to establish effective measures for allergy prevention (*24*). Also a quantitative risk assessment model for allergens based on probabilistic techniques instead of conventional deterministic approaches was suggested, in order to to obtain a more exhaustive risk assessment and more detailed information (*25*).

For bioinformatics comparison of proteins, three comparative approaches have traditionally been used or considered for safety evaluations:

1. Identifying any short segment (6-8 amino acids) of the protein that exactly matches a known allergen sequence.
2. An overall primary sequence comparison using Basic Local Alignment Search Tool (BLAST) or FASTA to find matches of greater than 35% identity over 80 amino acids.
3. Programs which identify 3-D similarities that might predict potential cross-reactivity.

The utility of each of these approaches was recently debated in a bioinformatics workshop (*26*). The consensus agreement from the expert participants in the workshop was that the short-segment match (*e. g.*, 6-8 amino acids) provides an unacceptably high rate of false positive matches and an uncertain rate of true positive matches, and was not particularly useful for an allergenicity evaluation performed in the context of a comprehensive safety evaluation. There was no consensus regarding the most appropriate bioinformatics method, an acceptable scoring criterion for triggering closer examination subsequent to a positive match, or an acceptable scoring mechanism for ranking the utility of the various 3-D approaches. However, the general consensus was that the most practical approach at this time to evaluate primary sequence identities to known allergens is using either FASTA or BLAST. While there was good agreement that identities of greater than 35% over 80 or more amino acids (recommended by CODEX in 2003) is quite conservative, the conclusion was that additional data or studies would be needed to justify changing this criterion as there is evidence that some individuals sensitized to proteins in evolutionarily conserved protein families may experience cross-reactions to proteins sharing approximately 40% identity (*26*).

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Chapter 22

Food and Respiratory Allergies in South India: An Overview

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Allergies especially food allergies are rarely documented in India even though they are highly prevalent. Insufficient clinical data is presently available regarding food allergies in south India and the allergen profile peculiar to Mangalore, a city in coastal India, has not been profiled so far.

The aim of this study was to estimate the incidence of food allergies in patients with rhino-bronchial allergy prevalent in Mangalore, a coastal city in south India.

Methods, Materials and Results

The study was carried out in a tertiary medical college teaching hospital in Mangalore, located in coastal belt of south India. One hundred consecutive patients suffering from rhino-bronchial allergy (RBA) were included. Rhino-bronchial allergy was confirmed by clinical evaluation, pulmonary function tests, chest radiographs, serum IgE estimation, and osteomeatal complex (OMC) computerized tomographic scans. All these patients were subjected to skin prick (SPT) which was done with standardized allergens as per standard recommendations. The skin tests kit included 86 different aeroallergens along with 26 common food allergens. The allergen categories were tree, weed, dust, insects, danders, food allergens, and miscellaneous allergens.

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Among one hundred patients suffering from rhino-bronchial allergy, the incidence of food allergy was 41%. Allergy to a single tested food allergen was seen in 23%, while 18% showed allergies to multiple food allergens. The prevalence of food allergies were highest to egg (8%), milk (8%), and green gram (8%). The prevalence of other food allergies tested in decreasing orders of their frequency were chick peas (6%), wheat (5%), pista (4%), black gram (4%), peanut (3%), mustard (3%), chocolate (3%), soya (3%), cashew nut (3%), walnut (2%), coffee (2%), banana (1%), and red gram (1%). The average total serum IgE was 1119.6 IU/mL in patients with RBA while in those with food allergies it was 1140 IU/mL and 1105 IU/mL in those with RBA without food allergies.

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Discussion

Allergy is a term often used 'to give a touch of mystification to ignorance'. This quote is specifically relevant to India, where allergy is highly prevalent although a grossly under-reported phenomenon. For the efficient diagnosis of the allergy and its effective treatment it is very important to know about the prevalence, seasonal and annual variations of aeroallergens, flora and fauna prevalent in the area, and the dietary profile characteristic of the local population.

The chest & allergy clinic in the tertiary hospital in Mangalore is attended by a diverse mix of patients from both urban settings and a sizeable rural population from the satellite villages around the city. In today's urban Mangalore, allergies due to aeroallergens, indoor air pollution by microorganisms such as fungal spores causing sick building syndromes, and food allergies are frequently reported. As has been previously proved, dust mites are an important source of inhalant allergens particularly in the coastal areas of the country and Mangalore is no exception. Urbanization with its high levels of pollution is linked to high incidence of pollen, dust, and microbial spore induced respiratory allergies, as it enhances the airway responsiveness to aeroallergens. Rolling of tobacco in beedis is an important occupational hazard as allergy to tobacco leaf dust and fungal contamination of the tobacco leaves in the cramped workplaces lead to pulmonary symptoms.

Food allergies often masquerade as other systemic diseases and often the symptoms are very subtle. The food allergy pattern of a country is influenced by the foods most commonly consumed. The dietary habits prevalent in Mangalore are quite diverse and are peculiar to this part of the world. In Mangalore, the majority of the population consumes a mixed diet made up of pulse (legumes), cereals, vegetables, and exotic sea foods, including fish. The prevalence of food allergies for a few tested allergens amongst patients with RBA was surprisingly high at 41%, with multiple allergens being implicated in 18% of these cases.

Allergy to cashew pollen (*Anacardium occidentale*) detected in 3% of RBA patients in this cashew growing belt often leads to asthma like symptoms (*1*). Also seen in this coastal belt with its strong inclination to seafoods are food allergies to shellfish, shrimp, oyster, and other exotic sea foods. We have been unable to document seafood allergies due to non-availability of standardized allergens. However quite a number of patients had historical and clinical evidence of seafood allergy. A higher incidence of other food allergies encountered in this population is also possibly linked to prior sensitization and cross reactivity to these seafood allergens. In contrast to many western countries, chickpea preparations are consumed in large quantities in this part of the world (*2*) and it is also reflected in our study which showed a high incidence of allergy to chickpeas. The predominant symptoms after chickpea ingestion were respiratory. Our study also showed moderate prevalence of allergy to wheat plant antigens (5%), with lesser prevalence of allergy to wheat threshing dust (WTD) and to wheat dust antigens, unlike studies reported in other parts of India which show lower incidence of allergies to wheat antigens (*3*). This interesting variation in clinical profile of allergy amongst the populace in this coastal city needs further validation by aerobiological studies. Also, the possible allergens need to be characterized biochemically and at a molecular epitope level for proper diagnosis and treatment of allergy. Further elaborate studies need to be done to characterize, diagnose, and treat food allergies.

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Chapter 23

European Survey for Hidden Allergens in Food: A Case Study with Peanut and Hazelnut

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During the EU-funded project Allergentest (QLK1-CT-2001-01151) a survey for the presence of hidden proteins of hazelnut and peanut in suspected pre-packed foodstuffs within EC member states was carried out to check the usefulness of the developed rapid test-kits. There were 11 participating countries in this study: Belgium, Portugal, Italy, Spain, France, United Kingdom, Slovenia, Greece, Norway, Czech Republic, and Austria. Each country submitted between 35-40 food samples. All foodstuffs were tested using the two test kits developed within the EC-project. The results presented were quantitative for the ELISA and qualitative for the LFD method (meaning positive or negative results - presence or absence)

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for peanut and hazelnut. The results were compared with the information given on the labels. Although at the time of the survey the new European Labelling directive (2003/89/EC) for allergens was not in force, the comparison showed whether the labelling was sufficient or not and where problems occured.

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Uncertainty as to the content of potentially allergenic foods constitutes a potential health risk to people suffering from food allergy or hypersensitivity if food is contaminated with that particular ingredient. The presence of allergenic proteins, such as peanut and hazelnut proteins, in certain foods may cause severe adverse reactions in allergic patients (*1*). Even trace amounts can result in fatal reactions. Among food allergy, peanut and hazelnut allergy is common and severe. Since the only effective measure for these individuals is avoidance, powerful analytical methods are required for the screening of trace amounts of potentially allergenic proteins in food samples (*2*).

The objective of the Allergentestproject with a duration of 3.5 yrs was the development and optimization of immunoanalytical testing systems for peanut and hazelnut proteins with allergenic potential. Therefore, the major goal of this project was the development of rapid easy-to-use immunochemical tests for the detection of traces of peanut and hazelnut proteins with allergenic potential in food (*3, 4*). The test systems developed were thoroughly validated, including a collaborative trial with all participants involved in the project. The rapid and easy-to-use test systems should allow for the surveillance and monitoring of food during production and enable avoidance of contamination (*5*). The new methods should also serve as useful tools for assessing contamination and risks of exposure for allergic individuals. The development of rapid protein assays, which can be used by producers, will serve to protect and promote the health of the consumer. This should also help to contribute to accurate labelling of products in the EU as suggested in the Codex Alimentarius Commission and to achieve greater confidence in labelled products (*6*).

The food categories included in the survey were from the same matrices used in the collaborative trial *i.e.*, dark chocolate, milk chocolate, cookies, ice-cream, yoghurt, and breakfast cereals. Products suspected to have undeclared peanut and/or hazelnut but carrying no declaration were preferably included. However, many products already carried "may contain …" statements on their labels.

Samples

A survey for the presence of peanut and hazelnut proteins in suspected foodstuffs sold to the consumers within EC member states should give the opportunity to check the usefulness of the developed test kits. The Consumer agencies from 11 different countries bought and distributed prepacked food samples to the participating laboratories of the study. All the samples were tested by ELISA (Enyzme-linked Immunosorbent Assay) and LFD (Lateral Flow Devices) for peanut (PN) and hazelnut (HN).

Each country purchased samples from the following 6 categories: milk chocolate, cookies, dark chocolate, breakfast cereals, ice cream, and yoghurt. The number of samples purchased from each country for each category is listed in Table I.

The samples were all code-numbered, and sent to the participating laboratories for analysis.

Table I. Number of Samples According to Country and Food Category

	Milk Choco.	*Cookies*	*Dark Choco.*	*Breakfast Cereals*	*Ice Cream*	*Yoghurt*	*No. of Samples*
Austria	15	15	-	4	-	5	**39**
Belgium	9	8	10	10	-	-	**37**
Czech Rep.	8	17	-	12	-	-	**37**
France	5	9	5	7	4	6	**36**
Greece	8	11	5	10	-	-	**34**
Italy	3	15	4	6	11	-	**36**
Norway	9	8	-	11	7	-	**35**
Portugal	7	11	6	10	-	-	**34**
Slovenia	11	14	2	8	2	2	**39**
Spain	-	10	-	7	8	11	**36**
UK	9	13	-	-	-	7	**20**
No. of Samples	**84**	**131**	**32**	**85**	**32**	**31**	**383**

Survey Results

Samples (383) were analysed according to the instructions given for the laboratory based ELISAs and for the LFDs. All samples were tested in duplicate for each test. The results were calculated, summarized, and compared with the labels.

In total 145 postitive samples were detected with 18 positives for peanut and 127 postives for hazelnut. Labels of these positive samples were compared (Table II) and from the data collected it was found that for peanut only 1 sample was positive where the label mentioned "may contain traces peanut and other nuts". Out of the 18 positive detected peanut samples 16 samples showed up positive with no declaration of peanut on the label.

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Table II. Positive Testeded Samples per Country and Comparison with Labelling

Positive Samples	*Peanut*	*Labelled may cont.*	*Unlabelled*	*Hazelnut*	*Labelled "may cont.*	*Unlabeled*	*Total Number of Samples*
Austria	4	1	3	21	3	5	**39**
Belgium	2	-	2	15	8	3	**37**
Czech Rep.	5	-	5	8	-	8	**37**
France	-	-	-	10	6	3	**36**
Greece	4	-	4	14	-	12	**34**
Italy	1	-	1	10	-	10	**36**
Norway	-	-	-	3	2	1	**35**
Portugal	-	-	-	10	-	10	**34**
Slovenia	2	-	1	27	3	20	**39**
Spain	-	-	-	2	-	2	**36**
UK	-	-	-	7	2	5	**20**
Total	**18**	**1**	**16**	**127**	**24**	**79**	**383**

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More problems were detected with hazelnut. Although for many of the samples tested the content of hazelnut was declared on the label and was measured, 24 of the 127 positive samples included on the label the remark "May contain traces of nuts..." and 79 samples were found to be positive for hazelnut without any declaration on the label. In the following results the undeclared samples are subdivided into peanut and hazelnut results according to food category and country.

Peanut Results

Table III shows the positive samples for peanut separated into the different food categories tested.

Out of the 383 samples in total 4% were found positive for undeclared peanut content. Figure 1 shows the calculated percentages of positive samples per country and per category. No problems could be detected within the food categories ice cream and yoghurt. In total 3% positives were found in Italy (33% positive milk chocolate) and Slovenia (9% positive milk chocolate). In Austria 10% positive peanut samples were detected, with 7% positives found in milk chocolate and 20% in cookies. Belgium showed in total 5% positives with 11%

Table III. Peanut Positive Samples with Undeclared Peanut Content

	Milk Choco.	*Cookies*	*Dark Choco.*	*Breakfast Cereals*	*Ice Cream*	*Yoghurt*	***No. Positive Samples/ Total***
Austria	1	3	-	-	-	-	**4/39**
Belgium	1	-	1	-	-	-	**2/37**
Czech Rep.	1	-	-	4	-	-	**5/37**
France	-	-	-	-	-	-	**0/36**
Greece	2	1	1	-	-	-	**4/34**
Italy	1	-	-	-	-	-	**1/36**
Norway	-	-	-	-	-	-	**0/35**
Portugal	-	-	-	-	-	-	**0/34**
Slovenia	1	-	-	-	-	-	**1/39**
Spain	-	-	-	-	-	-	**0/36**
UK	-	-	-	-	-	-	**0/20**
No. Positive Samples	**7**	**4**	**2**	**4**	**0**	**0**	**17/383**

positive milk chocolates and 10% in dark chocolate. France, Norway, Portugal, Spain, and the UK had no problems with undeclared peanut contents. The most problems with undeclared peanut content were encountered in Greece (total 12%; 25% milk chocolate, 9% cookies, 20% dark chocolate) and in the Czech Republic (total 14%; 13% milk chocolate, 33% breakfast cereals).

Hazelnut Results

Table IV shows the positive samples for hazelnut where the content was not declared on the label, separated into the different food categories per country.

For hazelnut there were even more problems with undeclared contents. Out of the 383 samples, 20% were determined to be positive with no hazelnut labelled. Below 10% positives were only found in Belgium (8%), France (8%), Norway (3%), Spain (6%), and the United Kingdom (10%). Austria followed with 15% positives. Italy and the Czech Republic had 22% positive findings. Over 30 percent positives were found in Portugal (32%) and Greece (35%). And the highest percentage of undeclared positive hazelnut samples was found in Slovenia (51%). Comparing the affected food categories in Austria and France only the cookies were a problem (Austria 40%, France 33%). In Norway 9% positives were detected only in the breakfast cereals. In Belgium there were 20%

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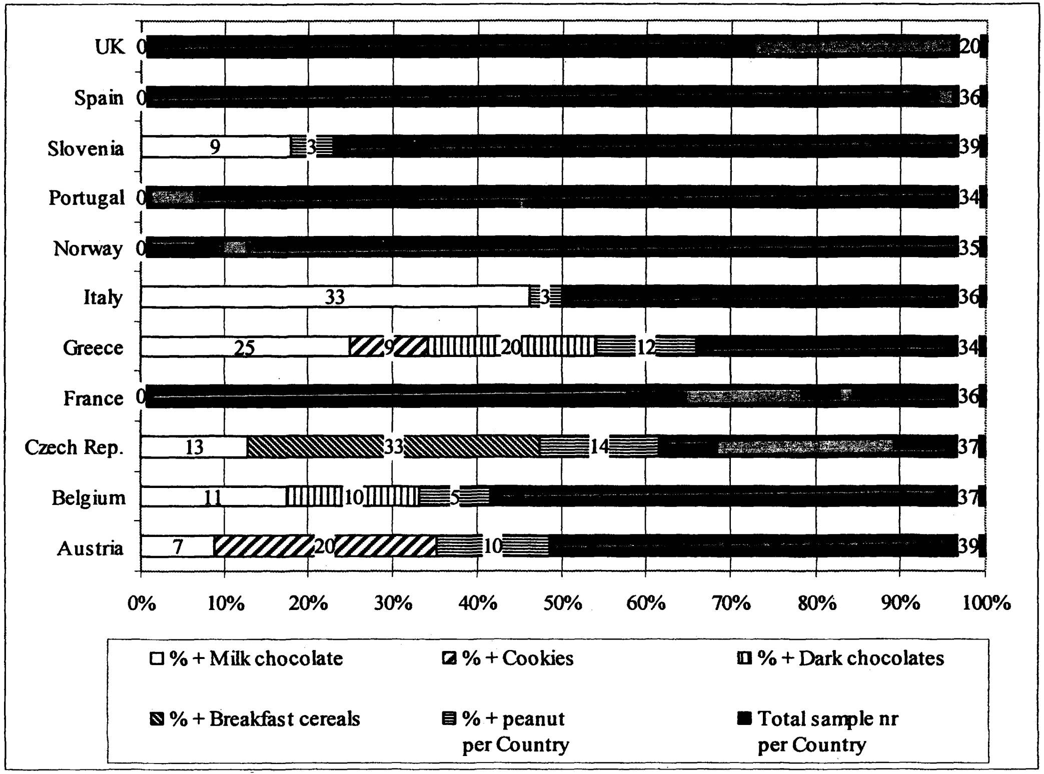

Figure 1. Positive samples (undeclared peanut detected) given as percentage of the number of samples per country and category.

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Table IV. Hazelnut Positive Results Detecting Undeclared Hazelnut

	Milk Choco.	*Cookies*	*Dark Choco.*	*Breakfast Cereals*	*Ice Cream*	*Yoghurt*	***No. Positive Samples/ Total***
Austria	-	6	-	-	-	-	**6/39**
Belgium	-	-	2	1	-	-	**3/37**
Czech Rep.	4	2	-	2	-	-	**8/37**
France	-	3	-	-	-	-	**3/36**
Greece	5	2	5	-	-	-	**12/34**
Italy	2	2	4	-	2	-	**8/36**
Norway	-	-	-	1	-	-	**1/35**
Portugal	4	3	4	-	-	-	**11/34**
Slovenia	4	11	2	3	-	-	**20/39**
Spain	-	-	-	-	1	1	**2/36**
UK	3	2	-	-	-	-	**2/20**
No. Positive Samples	**22**	**31**	**17**	**7**	**3**	**1**	**76/383**

positive dark chocolates and 10% positive breakfast cereals. No dark chocolate was collected in Spain but interestingly 13% positives were measured in ice-cream and 9% in the yoghurt samples. In the United Kingdom 15% positives in the category cookies were found and 33% of the milk chocolates contained undeclared hazelnuts. The milk chocolate was also a problem in the Czech Republic (50%), Greece (63%), Italy (67%), Portugal (57%), and Slovenia (36%). Also cookies were detected positive (Czech Republic 12%, Greece 18%, Italy 13%, Portugal 27%, and Slovenia 79%). Seventeen % breakfast cereals were found to be positive in the Czech Republic and 38% in Slovenia. In Italy ice cream seemed to contain undeclared hazelnut (18%) and dark chocolate was also found to be a problem. In Portugal, 67% positives were found and in Greece, Italy, and Slovenia all of the dark chocolate samples tested contained undeclared hazelnuts (Figure 2).

Summary

Figure 3 shows a comparison of the the countries for positive detected peanut and hazelnut content; no differentiation between the food categories was done.

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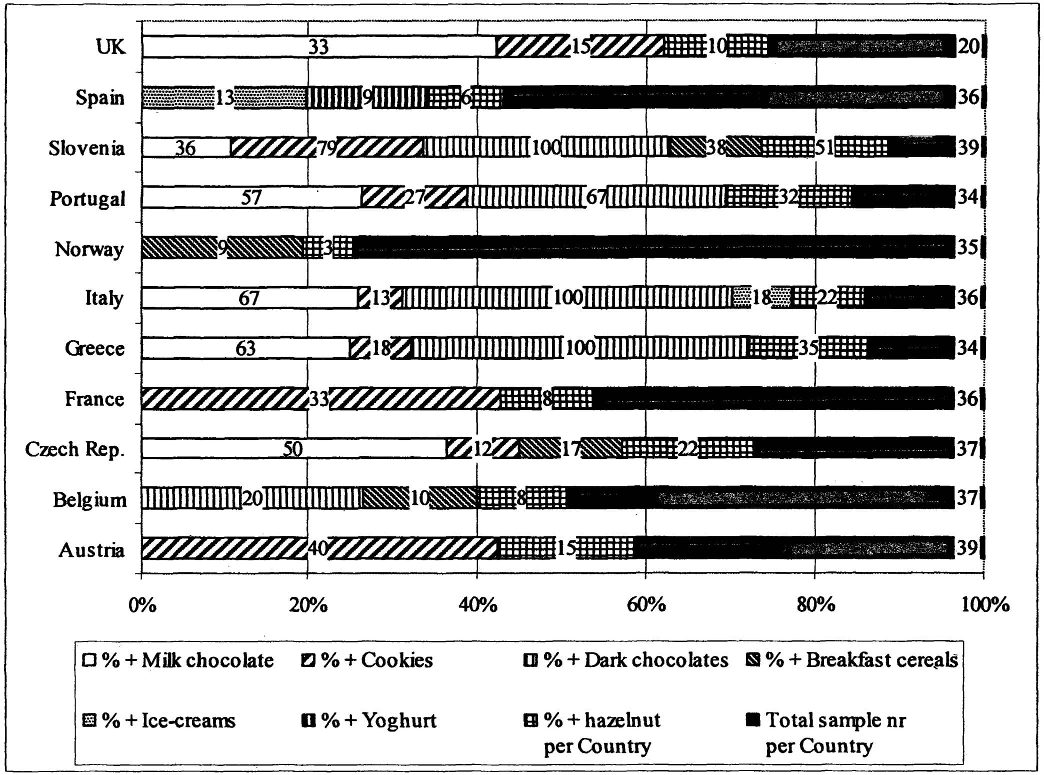

Figure 2. Positive samples (undeclared hazelnut detected) given as a percentage of the number of samples per country and category.

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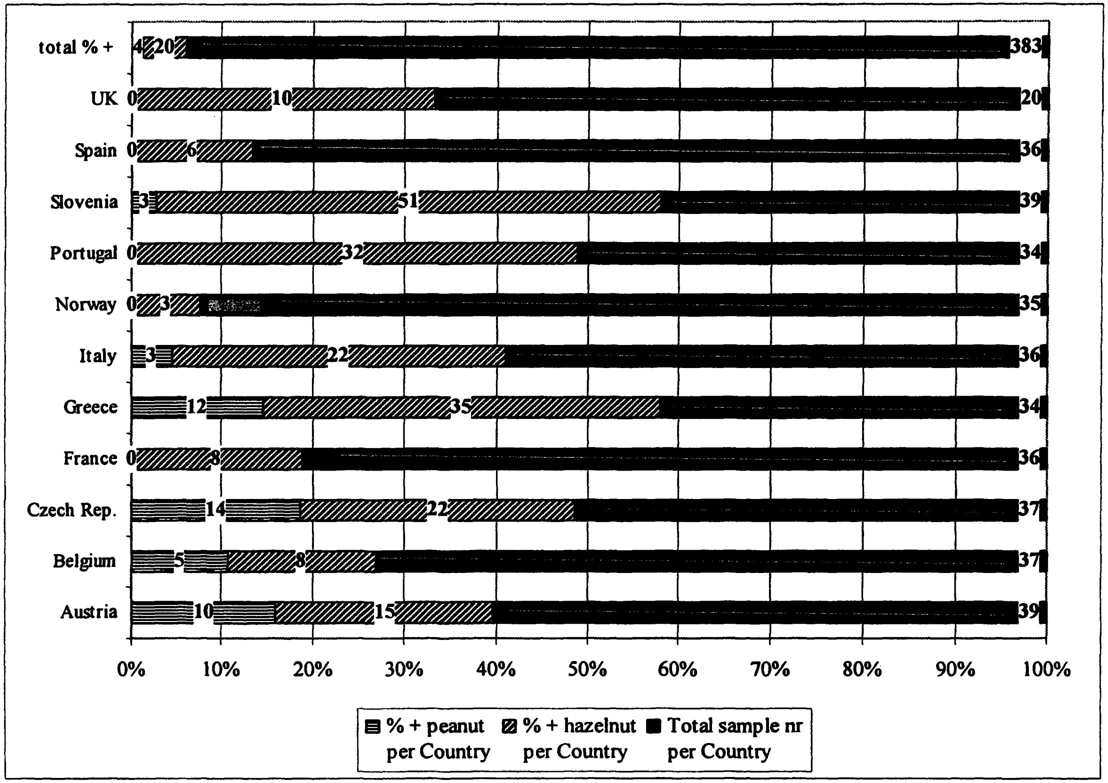

Figure 3. Percentage of samples positive for undeclared peanut and hazelnut, according to countries and in total.

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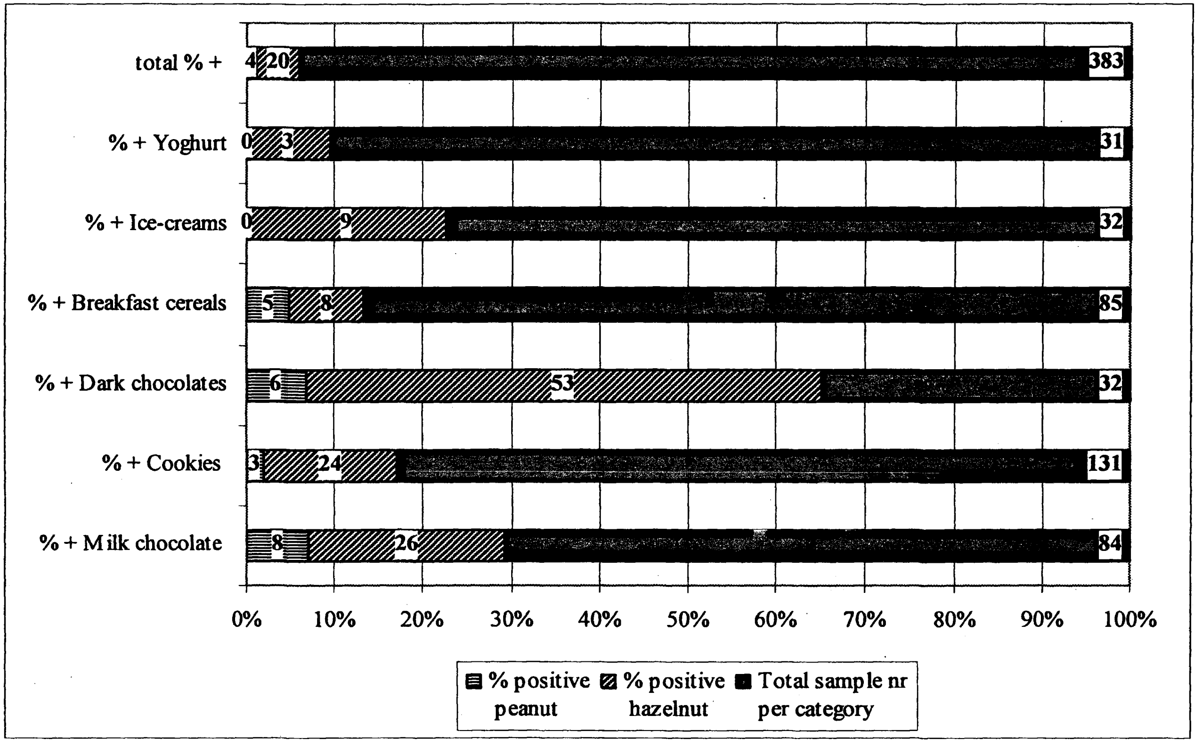

Figure 4. Percentage of samples found positive for undeclared peanut and hazelnut in 8 food categories.

Figure 4 shows the positive undeclared percentages of peanut and hazelnut for the measured food categories.

As a general conclusion from the Food Survey it can be said that peanut and hazelnut are widely present in a great number of foods and unfortunately they sometimes appear in foods where they are not expected to occur and are not labelled. Results showed that especially dark chocolate is a problem since around 53% were found positive followed by milk chocolate with 26% positives. This can pose a problem for allergic consumers.

In some countries, such as Slovenia, the positives were much more of a problem. One conclusion for this survey was that there are still great possibilities for the presence of undeclared ingredients (peanut, hazelnut, and in some cases both) and one has to ask the question whether this is due to intentional addition with insufficient labelling or/and accidental contamination. The results from France, Norway, Portugal, Spain, and the UK showed that in these countries only problems with hazelnut occured.

Hazelnut was found in many more cases than peanut (nearly 5 times mores hazelnut positive results than peanut); out of 383 samples 4% were found positive for peanuts and 20% positive for hazelnut. This is most likely due to European consumption habits and is in accordance with literature. In some countries such as France, Norway, Portugal, Spain, and the UK, only hazelnut was found. Food items such as cookies, chocolates, breakfast cereals, yoghurt, and ice-cream were tested and the results showed that, in terms of food categories, dark chocolate was the foodstuff with the most undeclared ingredients (53%). Milk chocolate and cookies followed with 26% and 24%, respectively. The category ice cream ranked 4th, with 9%, whereas breakfast cereals (9%) and yoghurt (8%) were at the end of the list.

Although labelling, which is intended for consumers in general, should not be regarded as the only medium of information acting as a substitute for the medical establishment, it is nevertheless advisable to assist consumers who have allergies or intolerances as much as possible by providing them with more comprehensible information on the composition of foodstuffs. Therefore, it is of the utmost importance that Directive 2003/89 be transposed into national law by all Member States (*7, 8, 9, 10*). Fortunately, almost all participating countries have done so, except for Italy at the moment.

Looking at the overall results, in terms of countries, it can be seen that all 11 countries showed positive results (although in some countries such as Slovenia this is much more of a problem). The presence of these undeclared ingredients (peanut, hazelnut, and in some cases both) reveals two possible situations: intentional addition with insufficient labelling or accidental contamination. In any case, and in order to achieve a high level of protection and guarantee consumers the right to information, producers have to be more careful when choosing their ingredients. An indication of the presence of these ingredients on their labels must be given in order for allergic individuals to avoid consuming

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potentially harmful allergens. Regular and adequate cleaning of production lines should be performed in order to avoid accidental contamination and allow correct labelling.

Finally, it is also important to keep this list under review in the light of changing food practices, emerging new clinical observations, and other kinds of scientific information. This would mean having the possibility of including and deleting certain ingredients and substances from the list based on scientific criteria that should be collected and thoroughly reviewed by the European Food Safety Authority (EFSA).

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Chapter 24

Molecular Allergology of Egg White Ovomucoid

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Allergens could be altered by genetic modifications to reduce their ability to initiate an allergic reaction. Ovomucoid is a dominant allergen in hen's egg-white. In the present study, we investigated the efficacy of a genetic variant of ovomucoid (GMFA) to modulate the allergic response in mice and characterized the role of GMFA in desensitizing ovomucoid-sensitized mice. The results suggest that GMFA could suppress allergic reactions in mice and desensitization with GMFA in ovomucoid-sensitized mice, significantly decreased allergic symptoms and histamine levels, suppressed production of ovomucoid-specific IgE antibodies, and modulated the T cell response, when compared to a control group treated with intact ovomucoid. This study indicates that GMFA can be used to potentially provide targeted immunotherapy for egg-allergic patients.

In Food Contaminants; Siantar, D., et al.;
ACS Symposium Series; American Chemical Society: Washington, DC, 2008.

Food allergy is an immunologic disease responsible for substantial morbidity and mortality. Food allergy occurs in 7–8% of children and 4% of adults (*1,2*) and the prevalence is increasing. Severe food allergic reactions may cause anaphylaxis and death. Food allergies cause approximately 30,000 anaphylactic episodes and 200 deaths per year in the U.S. According to the Asthma and Allergic Foundation of America (2002), allergies are the sixth leading cause of chronic diseases in the U.S. and the annual cost of dealing with them is estimated at $18 billion. Very often food-induced anaphylaxis following exposure to the ingestion of the so-called "hidden allergen" sources is the most common reason for someone with food allergy to report to a hospital emergency department with an anaphylactic reaction. While allergic reactions can occur with any food or food components, some are more common than others. Common food allergens include milk, egg, peanuts, tree nuts, wheat, fish, shellfish, and soy (*3*). Food allergens are mostly water-soluble glycoproteins that are 10 to 70 kDa (kilodalton) in size and fairly stable to heat, acid, and proteases.

Food allergic disorders encompass IgE-mediated hypersensitivity and cell-mediated hypersensitivity. Both immune mechanisms contribute to gastrointestinal (GI), cutaneous, respiratory, and systemic symptoms associated with food allergy. The GI tract is responsible for digestion and absorption of essential nutrients in the body. Delayed maturation of GI mucosal immunity and oral tolerance in newborns is linked to high incidence of food allergy in children under 5 years of age. Despite the evolution of the complex mucosal barrier in adults, about 2 % of ingested food antigens are absorbed and transported throughout the body in an intact form, even through the normal mature gut (*4*). Regional dietary habits and methods of food preparation play a role in the prevalence of specific food allergies. Since there are many hidden ingredients in food that could contain egg constituents and could trigger allergic reactions, an immediate need for treatment against egg-induced allergic reactions requires attention. The most effective strategy to prevent an allergic episode is strict food avoidance. Food avoidance is complicated by the ubiquitous distribution of peanut, soy, milk, egg, and other allergens in processed foods, poor food labeling practices, and cross-contamination of food products during processing. Hen's egg is one of the most commonly implicated cause of food allergies, representing the second major agent (30%) next to peanut (*5*). Type I allergic reactions to egg (IgE-antibody mediated) usually begin within minutes to one to two hours after ingestion. Eggs are composed of 56-61 % of egg white and 27-32 % of egg yolk. Egg yolk is considered less allergenic than the egg white (albumin) (*6*). Two-thirds of children diagnosed with food allergy are reactive to egg white (*7*). Allergic reactions to egg are more prevalent in children than in adults (*8*). Egg was reported as the cause of 11.6% of food induced anaphylaxis incidents (*9*). The symptoms associated with egg allergy include allergic rhinitis, asthma, dermatitis, diarrhea, gastrointestinal symptoms, hives, nausea, oral

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allergy syndrome, vomiting, wheezing, and, in some cases, anaphylaxis. Currently, there is no cure for egg-induced allergic reactions. Immune modulation offers the only opportunity to modify the underlying disease processes of allergy in a long-term cure, as no pharmacologic therapeutic agent has been shown to do this.

Avoidance may not be an explicit solution in case of egg allergy (*10*), due to the fact that eggs are important ingredients, associated with most of the food preparations. Foods containing eggs are very difficult to avoid, unlike peanuts, which could be avoided. Allergen-immunotherapy offers an alternative approach to controlling food allergy, by modulating the immune system. Immunotherapy may be used to treat food allergies by modifying the immune response to allergenic foods. Novel immunotherapeutic strategies include peptide immunotherapy, traditional Chinese medicine, mutated or homologous protein immunotherapy, DNA immunization, and immunization with immunostimulatory sequences, which all strive to elicit a decreased T helper cell type 2-like response or tolerance by the immune system in response to a specific food allergen (*11*).

The major egg white allergens are ovomucoid (Gal d1), ovalbumin (Gal d2), ovotransferrin (Gal d3), and lysozyme (Gal d4) (*12*). Ovalbumin was considered the major egg white allergen earlier (*13*) but recently ovomucoid has been shown to be the dominant allergen in hen's egg white (*14*). Ovomucoid is a highly glycosylated protein containing 20-25% carbohydrate, with a molecular weight of 28 kDa and an isoelectric point of 4.1 (*15*). It consists of 186 amino acids with three structurally independent tandem homologous domains (Domain I, II, and III) that are interconnected with peptide bonds. The domains could be cleaved by limited proteolysis or by cyanogen bromide or by a combination of both. Each domain is cross-linked within each other by intradomain disulfide bridges and behaves as a native globular protein, but there are no interdomain disulphide bridges (*16*). The carbohydrate moiety of ovomucoid seems to contribute to the thermal stability of its structure, since deglycosylation resulted in higher sensitivity to trypsin inhibition after heat denaturation (*17*). Carbohydrate moieties were reported to contribute to allergenicity of ovomucoid earlier (*18*), but in contrast they seem to have an inhibitory effect on IgE binding activity of ovomucoid, as shown recently (*19*).

The third domain of ovomucoid (DIII) was reported to be more allergenic with greater IgE binding activity than the first and the second domain using sera from egg allergic patients (*19*). Detailed B cell epitope mapping of the entire ovomucoid molecule has been reported and amino acids that are critical to IgE binding activity have been identified (*20-22*). Substitution of a single amino acid was shown to drastically reduce the ability to bind IgE, in the case of an apple allergen (*23*). A similar effect has been observed with the two site mutation of

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the ovomucoid gene (where glycine was substituted with methionine and phenylalanine was substituted with alanine (GMFA)), which led to complete loss of IgE response against sera from egg allergic patients (*24*).

Use of hypoallergenic formulas is an alternate strategy to avoid food allergies. Traditional allergy shots have not been proven practical in case of food allergic reactions, one of the disadvantages being the use of natural extracts which could trigger allergic reactions and cause severe side effects. Promising therapeutic modalities for the treatment and eventual prevention of food allergy are being developed and are in focus in recent years (*25*). Recent advances in food allergy research suggest that new research directions focused at the molecular level will advance our understanding of the mechanisms of food allergy and translate these findings into new options for identification and treatment of susceptible individuals. Conformational variants of allergens, displaying reduced allergenicity accompanied by retained IgG and T cell recognition, offer a safe, specific, and flexible approach to immunotherapy of type I allergy (*26*). The current principal approach to allergen modification is to modify B cell epitopes to prevent IgE binding, while preserving T cell epitopes to retain the capacity of inducing tolerance. In this way, the modified allergen will be directed to T cells by a phagocytosis-mediated antigen uptake mechanism, bypassing IgE cross-linking and IgE-dependent antigen presentation (*27*). Informative peptides that are predictive for the persistence of food allergic reactions have to be identified initially.

Vaccination with genetically engineered allergen was recently shown to prevent progression of allergic diseases (*28*). Many studies on allergen immunotherapy have shown modulation of T-cell response with inhibition of Th2 cell response (Interleukin (IL) -4) and induction of more Th1-like response with increased allergen-induced inteferon (IFN)-γ and interleukin-12 (*29*). A variety of avenues is being explored on the basis that many clinically important allergens have been identified, purified, cloned, epitope-mapped, produced as biologically active recombinant proteins, and administered safely by mucosal and cutaneous routes (*30*). Based on sequence information, protein engineering could be used to design homologous, nonallergenic proteins with similar structures, stability, and biologic/functional properties. The research work presented here describes characterization of a genetic variant of ovomucoid third domain (GMFA) in alteration of allergic response for immunotherapeutic treatment of egg-induced allergic reactions in a mouse model system. The present study was carried out to investigate possible mechanisms to suppress egg white allergy by a genetically modified variant of ovomucoid (GMFA) and to study the role of GMFA to alter and desensitize ovomucoid-induced allergic response in Balb/c mice.

Strategic Approaches for Specific Immunotherapy by Site-directed Mutagenesis

Allergen-specific immunotherapeutic studies and novel immunotherapeutic strategies designed to alter the immune response to food allergens are being examined as a potential treatment modality for food allergy (*31*). Successful immunotherapy is associated with a modulation of the immune response to allergens at the level of Th cells (*32, 33*). Recombinant allergens offer a safe alternative and can be modified to reduce the risk of IgE-mediated side effects (*34, 35*). Modified allergens should aim at the production of molecules with reduced IgE binding epitopes (hypoallergens), while preserving structural motifs necessary for T cell recognition (T cell epitopes) and for induction of IgG antibodies reacting with the natural allergen (blocking antibodies). Many hypoallergens in food have been studied for targeted protein modification, in order to alter protein function or properties in a predictable manner (*36-38*). Engineering of hypoallergens usually requires knowledge of B and T cell epitopes and in some cases of the three-dimensional structure of the native allergen (*39, 40*). The knowledge of the major IgE binding epitopes creates the possibility for a specific vaccination or immunotherapy in allergic patients. Targeted immunotherapy has been the only curative approach for type I allergies (*41, 42*). Most of the treatment procedures using specific immunotherapy are accompanied with the risk of IgE-mediated side effects and shows variable clinical efficacy. For example, initial trials of immunotherapy for food allergens have demonstrated an unacceptable safety to efficacy ratio (*43-45*). Molecular modification of food allergens are an alternative option for promising therapeutic modalities in the treatment and prevention of food allergy. The above mentioned results have prompted investigators to seek alternative forms of immunotherapy, as well as other forms of interventions for treatment of food allergy.

Modification of the IgE binding sites (epitopes) of allergens and production of hypoallergenic proteins are an alternative approach to attenuate hypersensitivity reactions for an improved immunotherapy of type I allergy. In this study we investigated the effect of the allergic response elicited by a genetic variant of the third domain of ovomucoid (GMFA), in comparison to the native third domain of ovomucoid without the carbohydrate chain (DIII-) in Balb/c mice. It was shown earlier that the engineered mutant (GMFA) had low IgE binding reactivity, upon replacement of the two critical IgE binding amino acids (G^{162} and F^{167}) on Western blot and ELISA, using sera from patients with egg allergy (*24*). The epitope sequence where the mutation was constructed was also predominantly recognized with Balb/c mice sera, raised against the recombinant wild type of the third domain of ovomucoid (*46*). We mapped the IgE binding epitopes of ovomucoid in mice and studied the effect of the mutant isoform (GMFA) in comparison to the native form of the allergen (DIII) in mice. We

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evaluated in detail, the allergen-specific immune response elicited in Balb/c mice by both the antigens, for specific antibody levels *in vivo*, and cytokine levels *in vitro*. These findings would draw attention to a potential use of this "hypoallergen" of ovomucoid as an agent in specific-immunotherapy to prevent egg allergy. To further evaluate if this "hypoallergen" was capable of desensitizing intact ovomucoid-sensitized mice, we characterized the potential role of the efficacy of this variant in desensitization of ovomucoid-allergic mice, using intraperitoneal injections. The clinical symptoms in the intact ovomucoid-sensitized (Fovm) and GMFA-desensitized animals were compared with control mice. Specific antibody and histamine levels were evaluated and the cellular response to both the sensitized and the desensitized groups were compared in order to analyze the efficacy of GMFA to inhibit allergic reactions.

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Mapping of IgE Binding Epitopes of Ovomucoid in Balb/c Mice

At least two IgE-binding epitopes are required to activate mast cells and basophils, via cross-linking of IgE bound to Fc_{ε}-receptors. In order to evaluate the immunological response of the modulated derivative of the ovomucoid gene in a murine model system (Balb/c), the IgE binding epitopes of ovomucoid in mice need to be identified. The T cell epitope mapping of ovomucoid in mice has been recently reported (*47*). Arrays of synthetic peptides representing the entire ovomucoid gene were synthesised on a derivatised cellulose membrane (SPOTs Kit, Genosys Biotechnologies, the Woodlands, TX) by Fmoc chemistry (using *N*-9-fluorenylmethoxycarbonyl chloroformate) as previously described (*22*). The peptides synthesized were 10 amino acids long and had an offset of 5 amino acids. The membrane was visualized under a light imager (EG & G Berthold, Bad Wildbad, Germany). The image processing was done using Win Light software (EG & G Berthold).

The obtained results demonstrated that the linear B-cell epitope recognized by the BALB/c mice, corresponds to the following sequence from the second domain – **GKVMVLCNRAFNPVC** and **YGNKCNFCNAVVESN** from the third domain as shown in Figure 1. Two adjacent epitopes were recognized by the sera from the BALB/c mice in the second domain (II) region and two major epitopes were recognized in the third domain (III) region which were also adjacent to each other. Interestingly, the major epitope which is recognized by the Balb/c mice in the third domain, was also the major epitope recognized by the egg allergic human patient's sera. Substitution of the critical amino acid at this epitope in the third domain of ovomucoid at position 32, where glycine was substituted by methionine (G to M) and phenylalanine at position 37 was substituted by alanine (F to A), led to complete loss of activity of IgE binding with patient's sera. The signal (relative intensity) of the spot was very strong with the epitopes in the third domain of ovomucoid and were very faint with the epitopes in the second domain, which agrees with our previous data (*19*) that the third domain is more allergenic than the first and the second domains. Incubation with normal murine serum alone revealed no non-specific binding of the murine immunoglobulins or of the secondary antibodies to the peptide sequences.

AEVDCSRFPNATDKEGKDVLVCNKDLRPIC GTDGVTYNNE
CLLCAYSIEF GTNISKEHDG ECKETVPMNC SSYANTTSED
GKVMVLCNRAFNPVCGTDGVTYDNECLLCAHKVEQGAS
VDKRHDGECRKELAAVSVDCSEYPKPDCTAEDRPLCGSDN
KT**YGNKCNFCNA VVESN**GTLTL SHFGKC

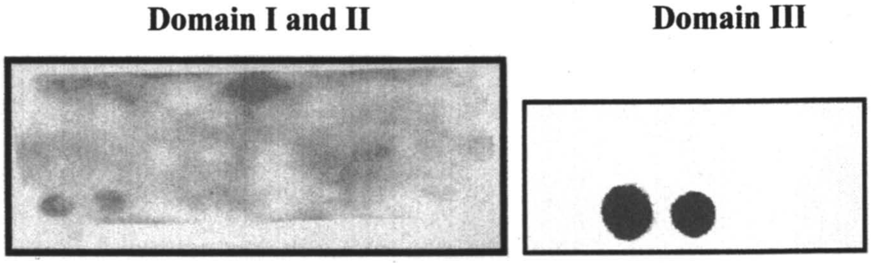

Figure 1. IgE epitope mapping of ovomucoid in mice sera. The epitopes identified in Balb/c mice are represented in bold and underlined on the primary sequence of the ovomucoid gene.

Animals, Experimental Design and Modulation of Allergic Response

Balb/c mice (four per group) (6-8 weeks old) were sensitized with the native third domain ovomucoid antigen without carbohydrate (DIII-), mutant ovomucoid antigen (GMFA), and PBS; 100 μg of the antigens were emulsified with 100 μL of complete Freund's adjuvant (CFA) and injected subcutaneously. Animals were frequently monitored for stress, pain, and lesions. Four weeks later, a booster was given with the same amount of antigens in Incomplete Freund's adjuvant (IFA). The control animals were immunized with PBS containing adjuvant. Two weeks later, all the animals were euthanized and blood samples and spleen were collected. Sera collected from mice at the end point were used to determine antigen-specific and total antibody levels. We evaluated

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in detail the allergen-specific immune response elicited in Balb/c mice by both the antigens. The total and specific IgE and IgG antibodies in mice sera were measured according to a previously described procedure (*48*). For specific IgG1 and IgG2a determinations, an indirect ELISA was performed. Sensitization of ovomucoid antigen DIII- in BALB/c mice resulted in high levels of specific IgE antibodies. On the contrary, the GMFA treated mice maintained significantly low serum specific IgE antibodies. Subtyping of the IgG class revealed that the mice immunized with the native third domain of ovomucoid (DIII-) showed detectable levels of specific IgG1, and low levels of specific IgG2a. On the contrary, in the sera sample collected from the mice immunized with GMFA protein, the specific levels of IgG1 were considerably lower when compared to the native third domain (DIII-), but a significant increase in the level of specific IgG2a was observed. GMFA was thus shown to inhibit IgG1 levels and up-regulate IgG2a production.

In order to determine if the IgE suppression effect of the mutant antigen had any influence on the cytokine profile, we determined the levels of Th1 and Th2 cytokines, secreted by the culture supernatants from splenocytes of sensitized mice (the native third domain ovomucoid (DIII), the mutant ovomucoid (GMFA), and the control group of mice). We detected elevated levels of IL-4 in mice sensitized with DIII antigen and significantly reduced levels of IL-4 in mice sensitized with GMFA. Considerable increase in the levels of INF-γ and IL-12 was observed with GMFA when compared to DIII antigen. This result suggests that the suppression effect of the mutant antigen (GMFA) is due to modulation of cytokines from an existing allergic Th2 to anti-allergic Th1 skewed pathway. To test whether IL-12 is involved in the IL-4–inhibitory activity of supernatants in GMFA samples, we examined the effect of neutralization of GMFA on the production of IL-4, INF-γ and IL-12, using different concentrations of monoclonal anti-IL-12 antibodies along with the control GMFA antigen to stimulate the culture supernatants. Addition of anti-IL-12 antibody abrogated the suppression of IL-4 in the case of the GMFA treated samples. The suppressed IL-4 levels were restored and INF-γ and IL-12 levels were suppressed with monoclonal anti-IL-12 antibodies tested at various concentrations. Our results show that the combination of the hypoallergen concept with the Th1-inducing approach, which is cytokine driven, would pave a way for therapeutic treatment of egg-allergy.

Desensitization (specific immunotherapy) helps in making an allergic substance less sensitive and is the only disease-modifying treatment or therapeutic option available today for a cure against allergic diseases, other than avoidance of a particular food. We have characterized and reported desensitization of allergic response to ovomucoid-sensitized mice. The rationale behind the use of a recombinant hypoallergenic variant of ovomucoid third domain described here is to modify the surface topography to reduce IgE binding activity, causing a partial disruption of α-helix, while retaining the folding

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pattern of the backbone to preserve surface structures capable of generating an IgG response. Loss of epitope for the specific IgE antibody and exclusive induction of Th1 cell differentiation and probable disruption of the α-helix in the tertiary structure of the third domain of ovomucoid, collectively make GMFA an ideal antigen for hyposensitization therapy. G32 which is located in β-bend of the third domain could have synergistic effect on antigenicity and F37, a core of the α-helix structure, has an important role in allergenicity; a combined effect of these two mutations causes complete abrogation of ovomucoid-induced allergic response. In order to dissect the underlying mechanism of protective response induced by the modified form of ovomucoid (GMFA) in desensitization, we analyzed the hypersensitivity symptoms of both the sensitized (Full Ovomucoid, FOvm) and the desensitized groups (sensitized with Fovm and desensitized with GMFA), along with the negative control groups (PBS). Acute symptoms (wheezing and breathing problems) scoring level 3 were observed in some mice sensitized with the intact ovomucoid antigen, whereas the desensitized group hardly showed any sign of hypersensitivity reaction except for slow movement in themice attributed to the injection pain (Figure 2A). Control groups treated with PBS did not show any symptom. We conclude that the modified ovomucoid (GMFA) protected our mouse model against an otherwise severe challenge of anaphylactic shock induced by intact ovomucoid in the desensitized group. Because histamine is one of the major mediators for anaphylactic reaction, we analyzed serum histamine levels in samples. Serum histamine secretion was blocked, indicating very low allergen-induced histamine release in desensitized mice, whereas intact ovomucoid sensitized mice showed significantly higher levels of serum histamine levels. The control group showed low or decreased levels of histamine (Figure 2B).

IgE plays a vital role in allergic reactions. Ovomucoid-antigen specific IgE antibodies were significantly reduced in desensitized mice ($P \leq 0.001$) when compared to the Fovm sensitized group. Specific IgE levels are direct indications for antigen-induced allergic response and it was significant from the data obtained that GMFA desensitization causes suppression of ovomucoid-specific-IgE antibodies, when compared to the high levels of specific IgE in the sensitized group. The PBS control sera elicited minimal or only very low antibody response (Figure 3A). IL-4, a T helper Th2 cytokine, regulates the switch from IgM/D to IgG1 and IgE in activated B cells (*49*).

Type 1 cytokines, such as interferon gamma (IFN-γ) and interleukin 12 (IL-12), steer the immune response towards a phenotype characterized by the production of immunoglobulin (Ig) isotype IgG2 in mice. Type 2 cytokines, such as IL-4 promote the production of Ig isotype IgG1. The Th1 cytokine IFN-γ is important *in vitro* and *in vivo* for enhancement of IgG2a secretion. Th1 and Th2 cytokines also function to cross-regulate Ig isotypes. For example, IFN-γ antagonizes IL-4-induced IgG1 responses at the level of IgG1 transcription (*50*), whereas IL-4 has the ability to suppress IFN-γ-driven IgG2a responses. The

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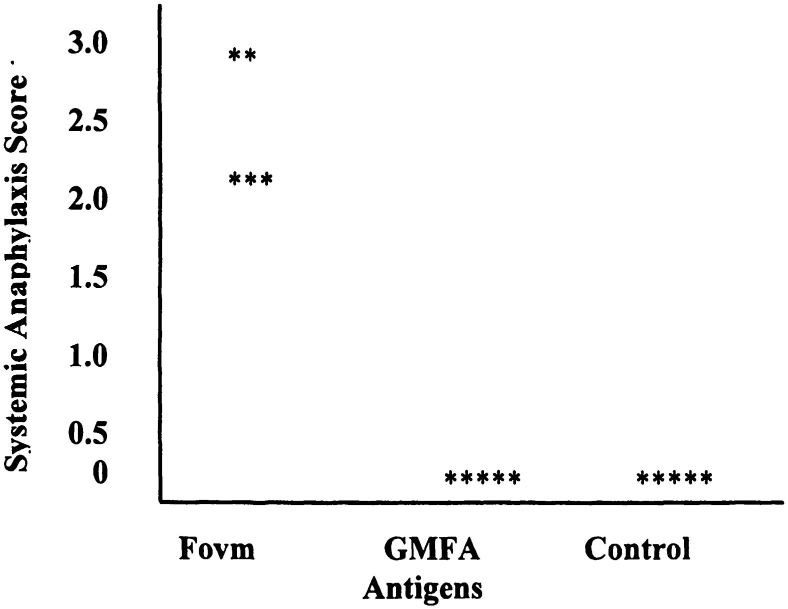

*Figure 2A. Score of the hypersensitivity signs. Mice were sensitized, desensitized, and challenged for induction of systemic anaphylaxis as described in Methods. Thirty to 40 minutes later, the symptoms of hypersensitivity were scored blinded on a scale from 0 (no symptoms) to 5 (death). Each * symbol indicates an individual mouse.*

isotyping of mice sera samples from the sensitized and the desensitized groups revealed that the IgG1 level was markedly increased in the intact ovomucoid sensitized group, which indicated a Th2 augmented reaction; the IgG2a level was enhanced in the desensitized group, reflecting a more Th1 favoring response in the desensitized group (Figure 3B). Control mice sensitized with PBS showed low levels of both IgG1 and IgG2a.

We hypothesized that desensitization with GMFA may promote antigen-specific Th1 response and consequently inhibit Th2 cytokine production. In order to test this hypothesis, we analyzed the levels of cytokines in culture supernatant of splenocyte samples from the sensitized and the desensitized group of mice. Spleen was isolated from individual mice, stimulated with intact ovomucoid antigen and incubated for 72 h. Cytokine levels were analyzed with reference to standard cytokines. It was observed that the desensitized group showed significantly low levels of IL-4 and increased levels of IFN-γ secretion in the culture supernatant samples, which were significantly different when compared against control sensitized mice, suggesting the occurrence of a discrete

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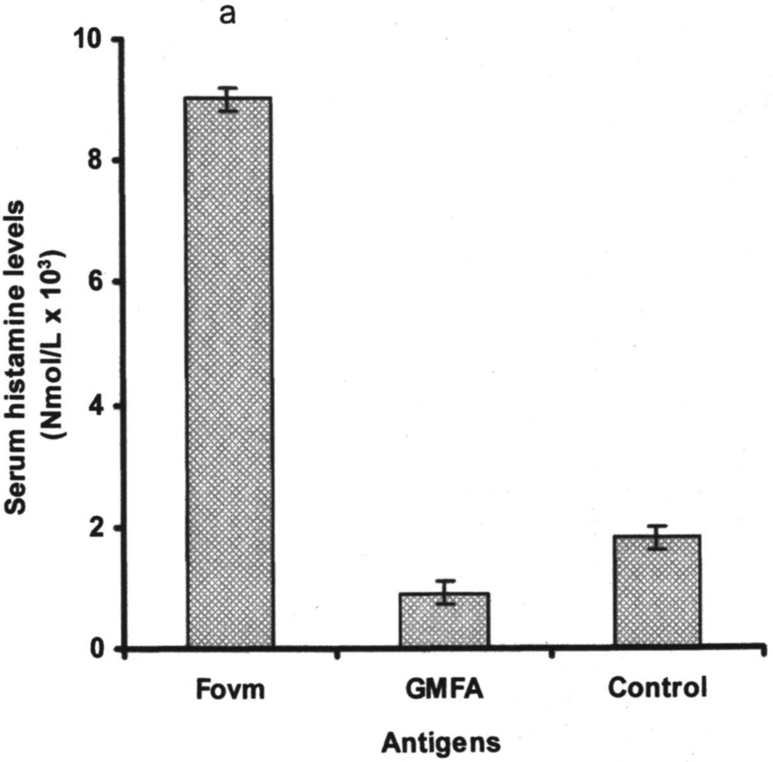

Figure 2B. Serum histamine levels in mice exposed to Fovm or GMFA antigens. The level of histamine was measured by inhibition ELISA and calculated in comparison to a standard curve. Values are expressed as mean ± SEM. Fovm results were significantly higher ($P \leq 0.001$) than results for GMFA and control groups.

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and prominent Th1 response, whereas significantly higher levels of IL-4 and lower levels of INF-γ were seen in the intact ovomucoid sensitized group (Figure 4A). Control group supernatant samples showed low levels of IL-4 and INF-γ production. The levels of IL-10 and IL-12 in culture supernatants were also quantified in comparison to standard cytokine curves (Figure 4B), to clarify whether GMFA desensitization induced these cytokines in vitro.

It was found that significantly higher levels of IL-10 and IL-12 were secreted in the desensitized group, as opposed to low levels of IL-10 and IL-12 in the sensitized group. The low levels of IL-4 found in the desensitized mice culture supernatant may be the result of an Ig-class switching attributed to the inhibition of specific IgE levels and bias towards a Th1 pathway. This

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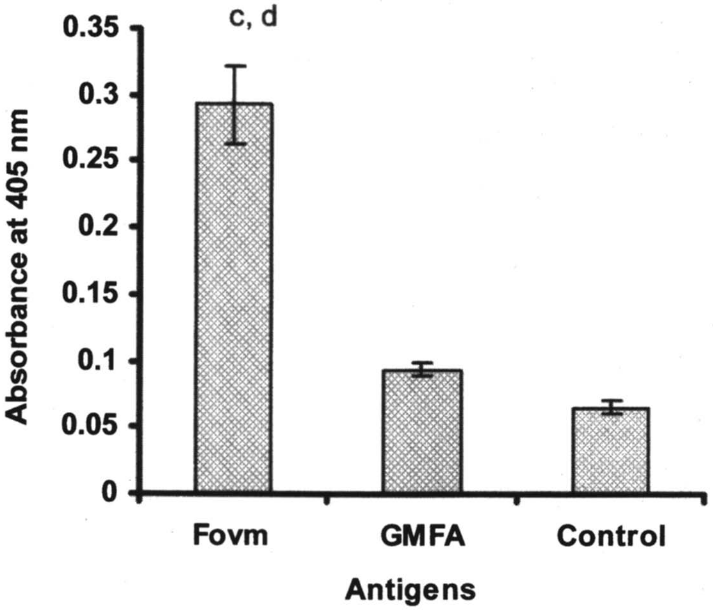

Figure 3A. Specific IgE levels in mice exposed to Fovm (Sensitized), GMFA (Desensitized), or Control (PBS) antigens. Levels of serum specific IgE were determined at the end point (day 42) in each individual mouse serum by ELISA. The specific levels of antibodies are represented as absorbance (OD) at 405 nm. Values are expressed as mean ± SEM. [c] *Significantly higher (P ≤ 0.001) than control group.* [d] *Significantly higher (P ≤ 0.001) than GMFA group.*

mechanism is further confirmed by high levels of INF-γ in culture supernatants of the desensitized group and low levels in the sensitized group. IL-12p70 is the most potent signal directing force for T cells towards an interferon γ-producing Th1 phenotype. Our study confirms this hypothesis and we observed an increase in IL-12 which could possibly be driving the production of INF-γ towards a Th1 bias in the desensitized group.

Conclusion

This research focused on characterization and evaluation of a hypoallergenic variant of ovomucoid third domain in suppression, and desensitization of ovomucoid-induced allergic reactions for immunotherapy. Egg allergies are the most common type of food allergy that occurs in people sensitive to one or more components of eggs. In such people, an allergic reaction occurs after coming into

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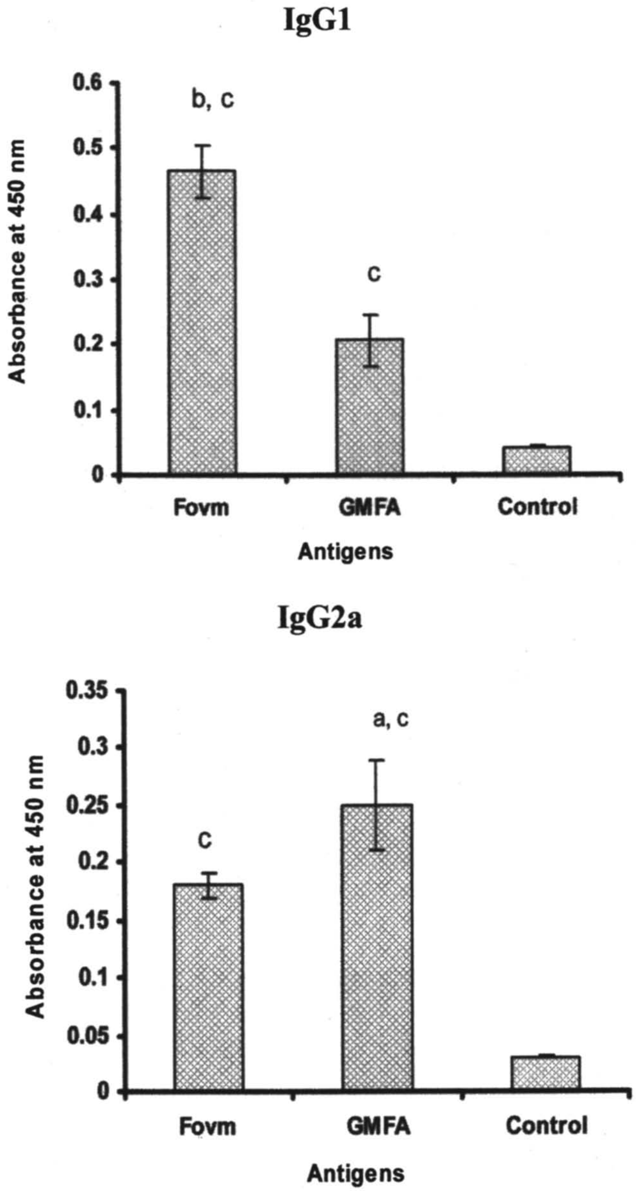

Figure 3B. Determination of specific IgG1 and IgG2a levels by ELISA in Fovm (Sensitized), GMFA (Desensitized), or Control (PBS) treated groups. Mice were sensitized, desensitized, and challenged and sera were collected and analyzed as described in Methods. Values are expressed as means ± SEM (n = 5 per group). [a] Significantly higher (P ≤ 0.01) than Fovm group. [b] Significantly higher (P ≤ 0.001) than GMFA group. [c] Significantly higher (P ≤ 0.001) than control group.

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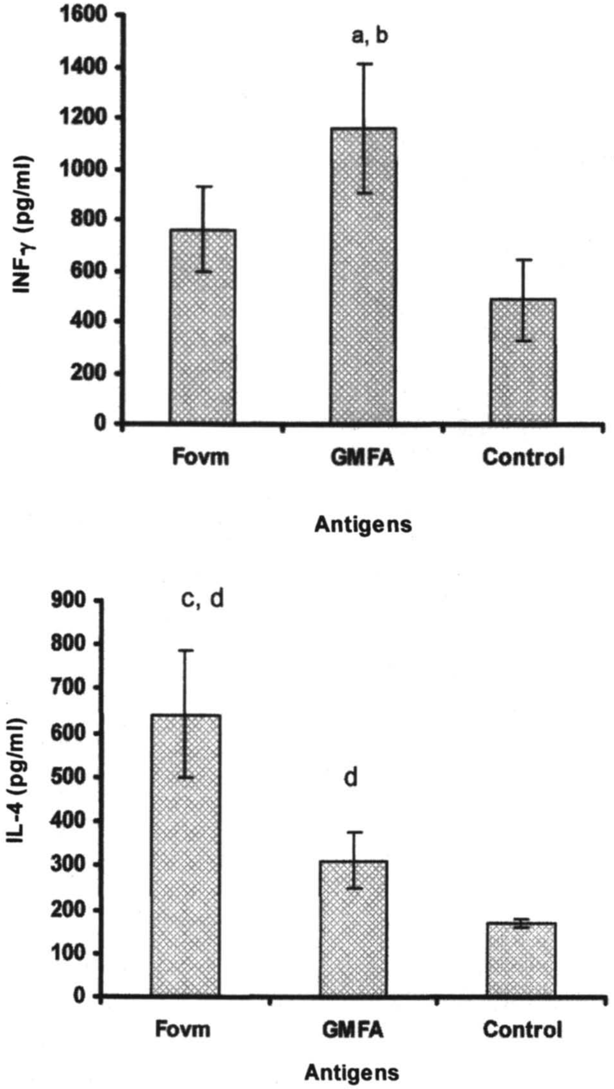

Figure 4A. Cytokine production in splenocytes from the sensitized, desensitized, and the control groups. Intact ovomucoid-activated splenocyte supernatants from all the three groups were collected and analyzed for the levels of IL-4 and INF-γ as described in Methods. Values are expressed as mean ± SEM of 5 mice. [a] Significantly higher (P ≤ 0.05) than Fovm group, [b] Significantly higher (P ≤ 0.01) than control group, [c] Significantly higher (P ≤ 0.001) than GMFA group. [d] Significantly higher (P ≤ 0.001) than control group. (Reproduced with permission from Clin. Exp. Immunol. ***2006****,* 145(3)*, 493-501. Copyright 2006 Wiley-Blackwell Publishers.)*

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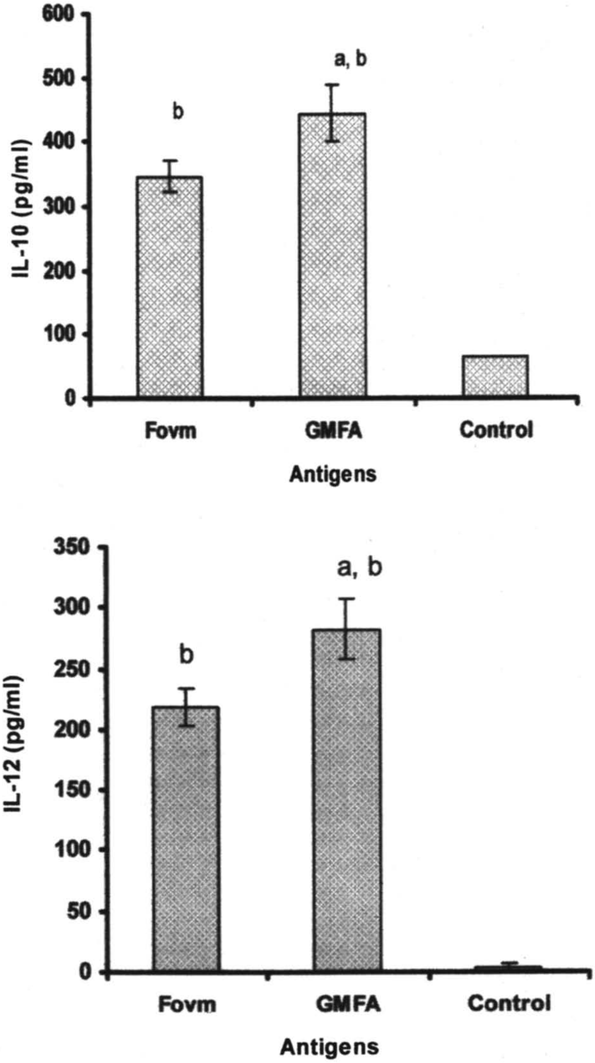

Figure 4B. Cytokine production in splenocytes from the sensitized, desensitized, and control groups. Intact ovomucoid-activated splenocyte supernatants were collected from all the three groups and analyzed for the levels of IL-10 and IL-12 as described in Methods. Values are expressed as mean ± SEM of 5 mice. [a] *Significantly higher (*$P \leq 0.01$*) than Fovm group,* [b] *Significantly higher (*$P \leq 0.001$*) than control group. (Reproduced with permission from* Clin. Exp. Immunol. ***2006****,* 145(3)*, 493-501. Copyright 2006 Wiley-Blackwell Publishers.)*

contact with eggs (usually by ingestion or consumption), resulting in allergic symptoms such as itchiness, rash, hives, stomach cramps, nausea, respiratory problems, and in some cases severe anaphylaxis. Most people with egg allergies are allergic to the egg white and ovomucoid is the most dominant allergen in the egg white. Since ovomucoid is responsible for most egg allergies, there is a need for prophylactic and therapeutic strategies for ovomucoid-induced allergic reactions.

Hypoallergenic mutants are potential candidates for future use in immunotherapy. They produce modified molecules with reduced IgE-binding epitopes (hypoallergens), while preserving structural motifs necessary for T cell recognition (T cell epitopes). Hypoallergens are designed by using structural information and knowledge of B and T cell epitopes of the allergen of interest. IgE-epitope mapping of ovomucoid in Balb/c mice was done and the amino acid sequence revealed homologous epitopes in both man and mice. This validated the Balb/c mouse system as a model for testing the response of GMFA and indicated the possibility, that it could be used in man as an immunotherapeutic vaccine.

In the present study, the results of screening GMFA and DIII for allergenic reactivity in mice revealed that GMFA suppressed allergic response in mice by lowering specific IgE levels, and changing an existing Th2 pathway to a Th1 skewed cellular pathway. Desensitization of ovomucoid-sensitized mice with GMFA revealed that this hypoallergen is capable of elimination of anaphylaxis symptoms, reducing histamine levels, lowering specific IgE levels, and modulating the allergen-specific Th2-dominated cellular response into a more Th1 phenotype, accompanied by enhanced production of IL-10. The present finding suggests that it is possible to develop a novel prophylactic and therapeutic form of hypoallergen for egg allergy for the prevention and/or reduction of allergic responses to ovomucoid.

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Chapter 25

Effect of Combined Use of High Pressure and Proteolytic Enzymes on Milk Allergens

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The application of high pressure during proteolysis is a very efficient way to produce whey protein hydrolysates with hypoallergenic properties, particularly for β-lactoglobulin, the major allergen in milk whey protein ingredients, and which is a very resistant protein to proteolysis.

Cow's Milk Allergy

Cow's milk allergy is the most common allergy in children, particularly in early childhood, with a prevalence of 2-3 % in infants during the first year of life, but this is outgrown in most cases at 3-4 years of age (*1*).Usually it is an IgE mediated reaction against cow's milk proteins that can cause cutaneous, respiratory, gastrointestinal problems, and, in severe but rare cases, anaphylaxis. It is manifested when cow's milk proteins are introduced in the diet through cow's milk, formulae based on cow's milk proteins, or even other products that contain milk protein ingredients.

The approximate milk protein composition of human milk, cow's milk and whey-based formulae is shown in Table I. All of the cow's milk proteins in the table are allergens in hypersensitive individuals, but caseins and β-lactoglobulin are considered major sensitizing agents (*2*). While cow's milk contains 80% caseins, human milk only has 40 %, being predominantly the whey proteins. β-Lactoglobulin, the major whey protein in cow's milk, is absent in human milk. Compared to human milk, cow's milk protein-based (or casein-based) formulae have a high content of caseins, but also contain β-lactoglobulin. On the other hand, whey based formulae, with similar proportions of caseins and whey

Table I. Protein Composition of Human Milk (HM), Cow's Milk (CM) and Whey-based Formulae (WF)

Protein	*HM*	*CM*	*WF*
Caseins	40	80	40
Whey proteins	60	20	60
β-Lactoglobulin	None	10	30
α-Lactalbumin	30	4	12
Bovine serum albumin	3	1	3
Lactoferrin	15	Tr.	1
Immunoglobulins	7	2	6

NOTE: Approximate values in percentage of total protein content. WF composition calculated for a casein/whey protein ratio equal to human milk.

proteins to human milk, contain high amounts of β-lactoglobulin. Therefore, these two proteins are the major challenges for children fed with infant formulae.

Hydrolyzed Formulae

Cow's milk allergy prevention and treatment rely on dietary hydrolyzed formulae, frequently based on caseins or whey proteins (*3*, *4*). Hypoantigenic formulae, or partially hydrolyzed formulae, can be applied for allergy prevention, although this remains a controversial issue. For infants already sensitized, dietary treatment is based on allergen avoidance, thus hypoallergenic formulae, or extensively hydrolyzed formulae, are used because of their good tolerance properties. The difference between these two types of hydrolysates is the molecular weight of their peptide components, which are smaller in the extensively hydrolyzed formulae. However, extensive hydrolysates may still contain large fragments and even intact proteins; this is the reason why, in many cases, they are subjected to an ultrafiltration process in order to remove the large peptide material.

Extensive hydrolysis is an efficient way to remove allergenic epitopes but it is detrimental to the organoleptic and functional characteristics. In order to improve hypoallergenic hydrolysates it is important to minimize the degradation of the peptides. Several authors have studied the allergenicity of whey protein hydrolysates in relation to the peptide size. Van Berejstein *et al.* (*5*) have shown that the minimum peptide size for eliciting allergenicity is 3000-5000 Da. Ena *et al.* (*6*) found that peptides with molecular mass lower than 3400 Da did not bind IgE from patients' sera. Van Hoeyveld *et al.* (*7*) reported that peptides above 2600 Da provoked positive skin response and IgE inhibition but peptides below 1400 Da did not provoke skin response although they were positive in IgE inhibition tests. The importance of the enzyme specificity in the manufacture of

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hydrolysates has also been pointed out (*6, 8*) and the use of selected fractions of the hydrolysates has been proposed to produce a well-tolerated formula (*9*).

β-Lactoglobulin

Whey protein-based hypoallergenic formulae have β-lactoglobulin as the major protein substrate. β-lactoglobulin is a small globular and compact protein, with known 3D structure (*10, 11*) (Figure 1) and it has been extensively studied (*12*). In principle, the absence of β-lactoglobulin in humans leads to a potential immune reaction towards epitopes on the whole protein. However, some protein regions have higher immunoreactivity. Several authors have studied the IgE binding capacity of different regions of β-lactoglobulin using tryptic and synthetic peptides (*13-15*). They found common epitopes for fragments 41-60, 95-113, 97-108, 102-124, and 149-162 that react with most patients' sera. In addition to these, other sequences that show immunoreactivity towards patients' sera cover most of the protein sequence. Fritsche *et al.* (*16*) have shown in sensitized rats that, among tryptic peptides, the sequence 149-162 retains the bivalent IgE epitope with highest mast cell triggering capacity.

In general, β-lactoglobulin is resistant to proteolysis and, in particular, to enzymes that target hydrophobic residues, *e.g.*, chymotrypsin and pepsin, and to those that work in acidic conditions, such as pepsin, due to the structural stability of the protein at low pHs (*17, 18*). Due to its relevance in hypoallergenic hydrolysates, an important issue is to enhance its proteolysis.

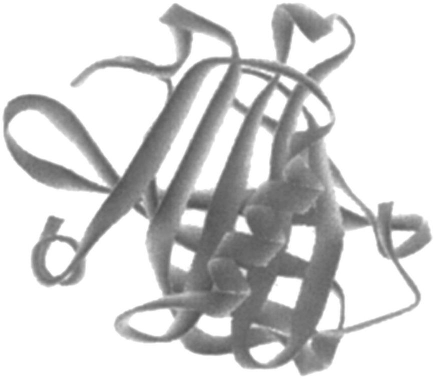

Figure 1. Structure of β-lactoglobulin.

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Proteolysis Enhancement of β-Lactoglobulin by Changing its Structure

The proteolysis process depends on the enzyme activity and the substrate structural state, since the enzyme attacks the protein on regions that are unfolded or flexible. Different methods have been used to modify the structure of β-lactoglobulin to make it more susceptible to the proteolysis attack, by unfolding the protein and exposing buried targets that otherwise would be inaccessible to the enzyme. Two major approaches have been tried: 1) modifying the protein before being hydrolyzed and 2) proteolyzing the protein while it is being unfolded.

Modification of β-lactoglobulin prior to the proteolysis process has been carried out by chemical means, such as esterification, causing a rapid hydrolysis by pepsin (*19*). However, physical treatments are usually preferred in order to maintain the amino acid sidechains unmodified. β-Lactoglobulin heated to temperatures higher than 85 °C is more susceptible to proteolysis by pepsin and trypsin than the native protein (*20, 21*). β-Lactoglobulin denatured by high pressure is also a better substrate for trypsin, chymotrypsin, and pepsin (*22-26*).

It has to be taken into account that physical treatments can cause reversible changes when mild conditions are used. When applied prior to proteolysis, it may lead to refolding of the protein when the denaturing conditions cease, burying again targets for the proteinase action. On the other hand, if the processing is intense, irreversible changes occur which can lead to the permanent unmasking of buried amino acids and also detrimental chemical changes. Heating leads to covalent binding of carbohydrates and loss of lysine through the Maillard reaction, as well as the formation of covalently linked proteins, mainly through S-S bonds (*27*). β-Lactoglobulin processed by high pressure undergoes aggregation through S-S exchange (*28*), but to a lesser extent than by heating (*29*).

On the other hand, proteolysis performed under denaturing conditions is very efficient, since proteolysis occurs while the protein is unfolded, and milder conditions can be used. Evidently, conditions have to be chosen so that the protein is unfolded and the proteinases maintain their activity. The addition of alcohols to a solution of β-lactoglobulin has been shown to modify the structure of the protein towards a α-helix structure and facilitate its proteolysis (*30*). Heating is an efficient way to proteolyse β-lactoglobulin (*31, 32*) and it is actually used in the manufacture of commercial hydrolysates. However, due to the secondary effects of heating, high pressure processing has become a very interesting alternative to heating for proteolysis enhancement (*22, 24-26, 33-35*).

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Proteolysis of β-Lactoglobulin under High Pressure

The effect of high pressure on β-lactoglobulin has been studied by various techniques (*36-52*). These studies have shown that the protein undergoes reversible structural changes that include dissociation, unfolding, and irreversible changes such as aggregation through SS/SH exchange reactions. Protein dissociation and the formation of a swollen state occur at pressures between 100 and 200 MPa and significant increase in the flexibility of the overall protein has been shown at pressures ≥200 MPa.

Recent studies in our laboratory (*25, 26*) have applied high pressure to study the proteolysis of β-lactoglobulin by trypsin and chymotrypsin. Similarly to other authors (*22, 24, 33, 35*), it was found that the proteolysis rate with trypsin and chymotrypsin increased significantly as pressure increased from 100 to 400 MPa, and it was particularly efficient at pressures of ≥200 MPa. This efficiency has been attributed to the pressure-induced exposure of ample regions of the protein (*24*). Proteolysis products originating from S-S bonds rearrangement have not been found or have been found in small amounts (*22, 24, 25*). Since no S-S rearrangements seem to occur in the peptides once released (*22*) it has been proposed that the proteolytic events precede the S-S/SH exchange reaction and, although a small amount of S-S oligomers may be formed, they would be readily proteolysed (*22, 24, 25*).

Analysis of the peptide pattern obtained in hydrolysates prepared at different pressures and times has shown that either at atmospheric pressure or at high pressure the same proteolytic products are obtained, but the quantities vary to different extents (*22, 24-26, 33*). For a similar degree of protein elimination, large peptides accumulate in the hydrolysates obtained under high pressure, compared to those obtained at atmospheric pressure, as illustrated in Figure 2. These fragments are reduced to smaller proteolytic products on longer incubation with the enzyme. Similar results have been found for β-lactoglobulin proteolysed under heat treatment (*31*). A progressive mechanism for proteolysis occurs at atmospheric and high pressure. The main difference is that at high pressure, the first steps are fast and the proteolysis into smaller fragments is slow, while at atmospheric pressure both processes are slow.

The overall effect of high pressure on the structure and proteolysis of β-lactoglobulin is depicted in Figure 3. Due to its compact structure at atmospheric pressure it is slowly proteolysed to large fragments that are broken up slowly into small peptides. Therefore, after some time, a rather inhomogeneous mixture is present, since by the time that the small fragments are already formed, the intact protein is still being cleaved for the first time. On the other hand, as the result of structural modifications induced by high pressure, the first steps in the proteolysis are accelerated, leaving no intact protein, but releasing peptides that are further cleaved, though slowly into small peptides. Because of the rapid

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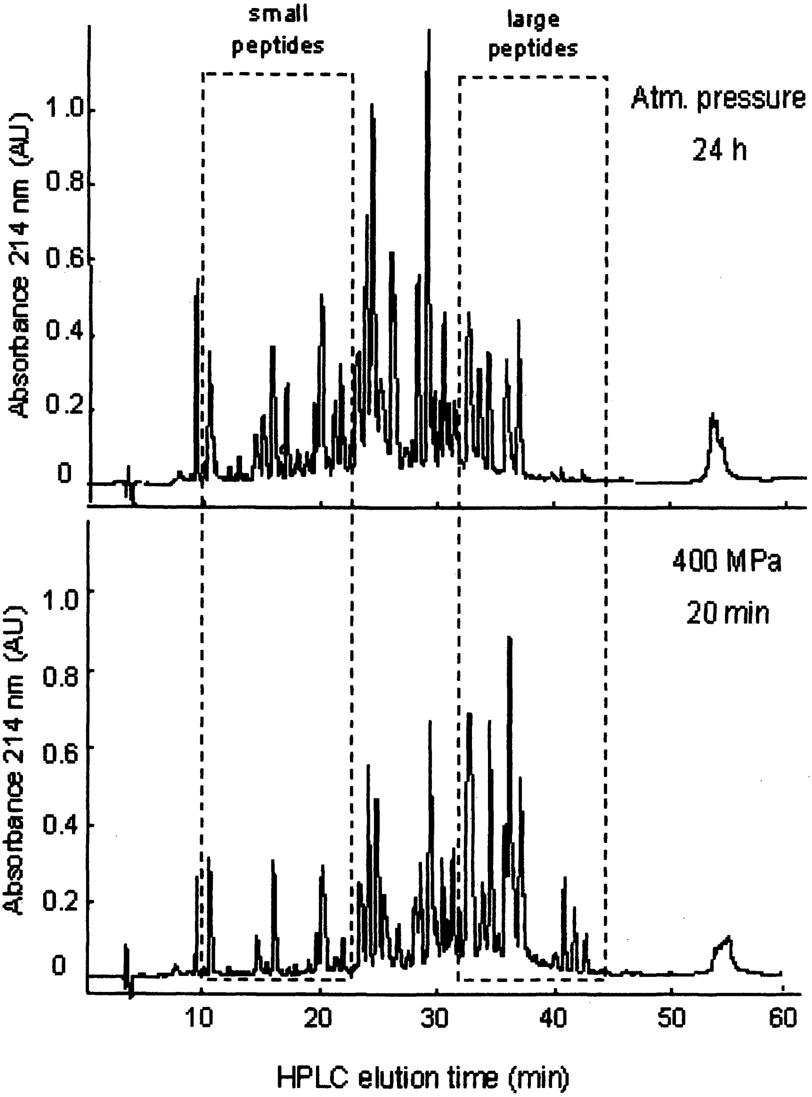

Figure 2. HPLC peptide profile of a chymotryptic hydrolysate of β-lactoglobulin obtained at amospheric pressure and high pressure.

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conversion of the protein into intermediate fragments, more homogeneity in the size of the peptides is expected.

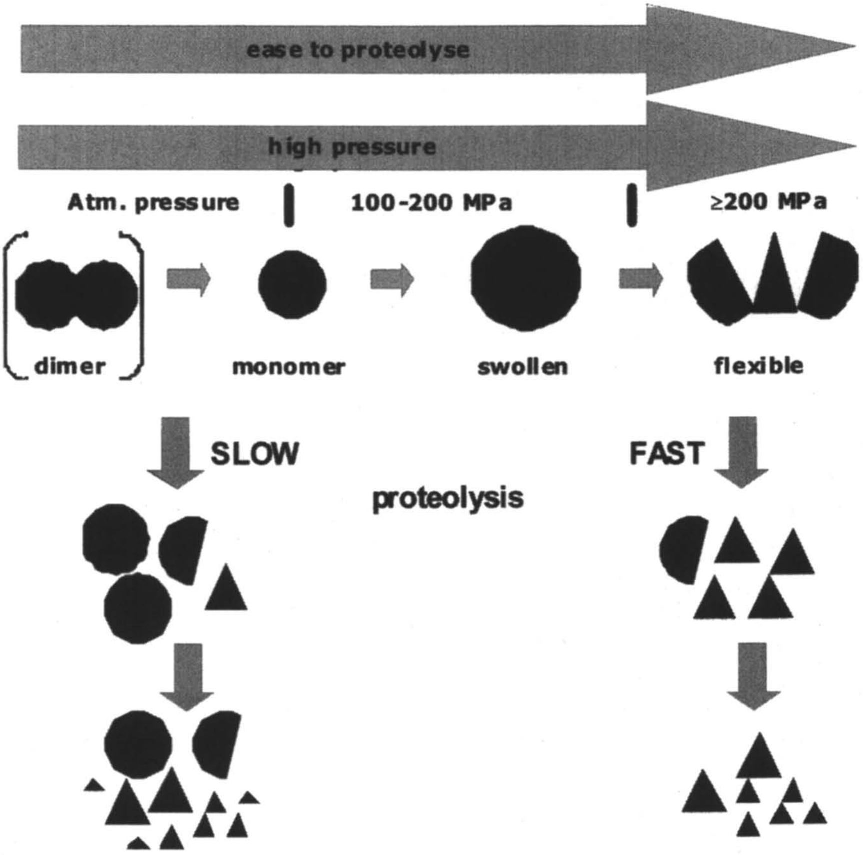
ease to proteolyse
high pressure
Atm. pressure
100-200 MPa
≥200 MPa
dimer
monomer
swollen
flexible
SLOW
FAST
proteolysis

Figure 3. Effect of high pressure on the structure of β-lactoglobulin and its proteolysis.

Hydrolysates obtained by enzymatic treatment of β-lactoglobulin under high pressure have shown reduced antigenicity and allergenicity, as assessed by the extent of binding to IgG antibodies raised against the protein, to IgE of sensitized animal sera, and to IgE antibodies of allergic patients' sera (*24, 53-56*). In accordance with these studies, preliminary results in our laboratory have shown that hydrolysis under high pressure for a short time (minutes) can remove most immunoreactivity against commercial polyclonal IgG and IgE of patients' sera and residual immunoreactivity can be removed by longer incubation time.

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Potential Application to the Manufacture of Hydrolysates

The most common method used for the production of protein hydrolysates is with a batch reactor, which uses proteinases while heating. Temperatures of about 50 °C and slightly alkaline conditions are used. The continuously stirred tank membrane reactor (CSTMR) incorporates an ultrafiltration system that allows recovery of the enzyme and selection of the molecular weights of the products. Enzyme leakage, membrane fouling, and inactivation of the enzyme by heat are the major drawbacks, although improvements have been made to overcome these effects (*57*).

Proteolysis under high pressure has some advantages over heating, such as energy saving and the possibility of manipulating pressure and temperature simultaneously. In addition, it causes minimal effects on flavor, color, nutrition, and protein aggregation (*29, 58*). The significant acceleration of the first steps of hydrolysis could be translated into a more efficient process. It is easy to obtain hypoallergenic hydrolysates with no intact protein and probably more homogeneous than those obtained at atmospheric pressure. The enzyme remains active after processing although the degree of recovery of activity should be tested. Whether this method is more efficient or cost saving than those currently used is not known but, in our opinion, it is worth to look into it further.

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Chapter 26

Evaluation of the Allergenic Potential of *Morinda citrifolia* L. (Noni) Leaf

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The allergenic potential of *Morinda citrifolia* L. (noni) leaf was evaluated in a pepsin resistance assay. Proteins extracted from the leaf were subjected to simulated gastric fluid at various incubation times, followed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), to determine their resistance to digestion by pepsin. Noni leaf proteins were readily digested at all incubation times. The results indicate noni leaf is not allergenic, as a lack of resistance to pepsin is a common characteristic of non-allergenic proteins. These data corroborate earlier studies of the leaf, demonstrating the potential for safe food use. This assay method may also be a useful tool in evaluating the safety of newly introduced tropical foods.

Morinda citrifolia L., more commonly known as noni, is distributed across the tropics and has large, evergreen, dark, glossy, prominently veined, elliptical to oblong leaves (*1*). Noni leaves have long been recognized as food among several cultures. They are included in the United Nations (UN), World Health Organization (WHO), and Food and Agriculture Organization (FAO) food composition tables for East Asia and the Islands of the Pacific (*2, 3*). Polynesians wrapped fish in noni leaves for cooking, and then ate them together (*4*). Indonesians also ate the leaves with fish, as a raw vegetable, or fruit side dish with rice known as "lalab", as a steamed vegetable, and as a vegetable-coconut-sambal dish called "oorab" (*5*). The consumption of noni leaves was so widespread in the Samut Prakarn province of Thailand that contamination of this plant by heavy metal emission from a local factory was considered a public health problem (*6*).

Noni leaves provide certain nutritional benefits. They have been investigated as a potential source of dietary protein in tropical areas where other sources may not be readily available (*7, 8*). The leaves are an excellent source of β-carotene, with a total carotene content higher than Chinese cabbage, taro, or any other green leaf produce commonly consumed in the Pacific. It was this property that resulted in the successful treatment of night blindness of children in the island nation of Kiribati (*9*). In addition to these nutritive properties, the phytochemicals have been found which exhibit significant antioxidant activity (*10, 11*).

Toxicity tests on the leaves began as early as 1965, when the LD_{50} of the methanol extract was found to be greater than 1000 mg/kg when injected intraperitoneally in male mice (*12*). The extract did not cause any symptoms of toxicity, including convulsions, diarrhea, tail erection, or exophthalmos.

Acute oral toxicity tests were also performed for hot water (MCW) and 50% ethanol (MCE) extracts in separate groups of 10 rats. All animals were observed for 14 days. No deaths nor adverse effects observed. Therefore, the LD_{50} for these extracts was greater than 2000 mg/kg (*13, 14*).

A second acute oral toxicity test of MCE and MCW was performed in separate groups of 50 mice (25 males and 25 females) at a dose of 2000 mg/kg. No deaths occurred, nor were other adverse effects seen. These results substantiated the LD_{50} of >2000 mg/kg in the rat studies (*15*). Additionally, a subacute (28 day) oral toxicity test of MCE and MCW in mice at 200 mg/kg (25 males and 25 females/test group) produced no evidence of toxicity (*16*).

A subchronic (90-day) oral toxicity study of MCW and MCE was also performed in mice. The extracts were examined in separate groups of 50 mice (25 males, 25 females) at 20 mg/animal (approx. 1000 mg/kg). Animals were observed 90 days for clinical symptoms, death, and body weight. No evidence of toxicity was observed (*17*). The no-observable-adverse-effect-level (NOAEL) of the extracts in rodents is greater than 2000 mg/kg.

While these toxicity tests reveal no inherent toxicity, they do not address the issue of allergenicity. With the ever increasing popularity of noni among populations where it is not part of the traditional diet, an assessment of the allergenic potential of the leaf is important. However, making this assessment presents several challenges.

The currently accepted and standard methods for investigating the potential for Types I (immediate) and IV (delayed) hypersensitivity reactions are not designed for ingested and chemically complex substances, such as foods. These assays involve sensitization and challenge, either via the cutaneous and inhalation routes, or by intravenous injection. Such methods involve non-

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physiological routes of exposure, in which digestion is not accounted for. Only very recently has there been some development using the brown Norway rat as a model for assessing the allergenicity of individual novel proteins (*18*). There also seems to be no generally accepted standard methods for assessing Types II (cytotoxic) and III (immune complex) hypersensitivities.

With new food sources, there is the disadvantage that epidemiological data to determine the sensitization rate is lacking. Further, there is often no previously sensitized subpopulation from which sera may be provided to conduct relevant Ig-E binding component studies. Some alternative methods for assessing the ability of a substance to sensitize susceptible individuals have been developed for recombinant proteins in genetically modified foods. However, these are only feasible when a small number of proteins are being investigated. These methods, such as protein profiling and comparison against databases containing amino acid sequences of allergens, are not an economically reasonable approach for new natural foods, where very large numbers of individual proteins may be involved.

With these limitations in mind, a modified active systemic anaphylaxis test in guinea pigs was performed, where the intravenous challenge of the test group was replaced with an oral challenge. None of the animals in the test group showed sings of acute systemic anaphylaxis (*19*). Injection of whole leaf, or even an aqueous extract, to sensitize the animals is inappropriate. These are either not readily absorbed (due to insoluble carbohydrates, such as cellulose and lignin) or may cause inflammatory reactions that are unrelated to oral sensitization (due to many high molecular weight soluble carbohydrates, of which carrageenan is an excellent example). Therefore, a freeze dried 80% ethanol extract of the leaf, with a protein content of 11.9%, was used as the test material, This allowed the extraction of ethanol-soluble proteins, similar to extraction of allergenic wheat gliadin peptides and storage proteins in rye and maize, etc. However, ethanol extraction may not include some proteins at the higher end of the molecular weight range of food allergens (*20*). Consequently, additional data are needed,which may be obtained from examination of a property common to food allergens. Allergenic proteins in food are resistant to digestion by the gastrointestinal tract. Conversely, proteins that are readily digested are not allergenic (*21*). The property of resistance to digestion may be evaluated *in vitro* and is useful in estimating the allergenicity of protein in novel foods (*22, 23*). Following the basic protocol of Astwood *et al.* (*21*), the resistance of *M. citrifolia* leaf proteins to digestion was evaluated in simulated gastric fluid (32% pepsin).

Material and Methods

Proteins were extracted from recently harvested *M. citrifolia* leaves with the Plant Total Protein Extraction Kit (Sigma®). The total protein content of the

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extract was determined using the Quick Start™ Bradford Protein Assay, with a bovine serum albumin standard (Bio-Rad Laboratories).

Soybean lipoxidase and trypsin inhibitor (both from Fluka) were used as reference controls. The total protein contents of these preparations were also determined by the Quick Start™ Bradford Protein Assay. A lipoxidase test solution was made with 15.39 mg in 2 mL Tris/NaCl buffer. Trypsin inhibitor solution was made with 26.55 mg in 2 mL Tris/NaCl buffer.

A 0.32% pepsin solution was prepared with 32.96 mg pepsin (2,580 units/mg) from porcine gastric mucosa (Sigma®) and 27.66 mg NaCl in 10 mL deionized water. This solution was adjusted to pH 2.07 with 0.1 N HCl.

The pepsin resistance test was carried out as follows: 50 μL aliquots of *M. citrifolia* leaf protein extract solution, lipoxidase solution, and trypsin inhibitor solution were each incubated in 200 μL of 0.32% pepsin solution at 37° C for 0, 15, 30, and 60 seconds. Immediately following incubation, each sample was neutralized with 75 μL of 160 mM Na_2CO_3 then heated at 96° C for 5 min.

All samples were evaluated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using precast 10-20% Tris-Tricine/Peptide Ready Gels (Bio-Rad Laboratories) and Tris/Tricine/SDS buffer (Bio-Rad Laboratories). Polypeptide molecular weight standards, as well as biotinylated broad range molecular weight standards (both from Bio-Rad Laboratories) were run with each gel, as well as the pepsin solution and sample solutions alone. Proteins were visualized by Coomassie brilliant blue staining.

Results and Discussion

The *M. citrifolia* leaf extract contained 1.91 mg protein/mL. The lipoxidase and trypsin inhibitor solutions contained 2.47 and 1.96 mg protein/mL, respectively. Therefore, the reference controls were appropriate for evaluation of the extracted leaf protein solution.

The SDS-PAGE results are presented in Figures 1-3. Trypsin inhibitor is a known allergen and is resistant to digestion. Soybean lipoxidase is a non-allergenic protein and is sensitive to pepsin digestion. Figures 1 and 2 demonstrate that our test system was suitable, as the trypsin inhibitor was stable throughout the incubation period, while the lipoxidase was degraded rapidly.

Figure 3 displays SDS-PAGE results for *M. citrifolia* leaf proteins, alone and in pepsin solution. Proteins from this leaf are very sensitive to the effects of pepsin. The proteins were degraded immediately, at 0 seconds, even with neutralization of the pepsin solution. Only protein from the simulated gastric fluid (0.32% pepsin solution) remained after any incubation time.

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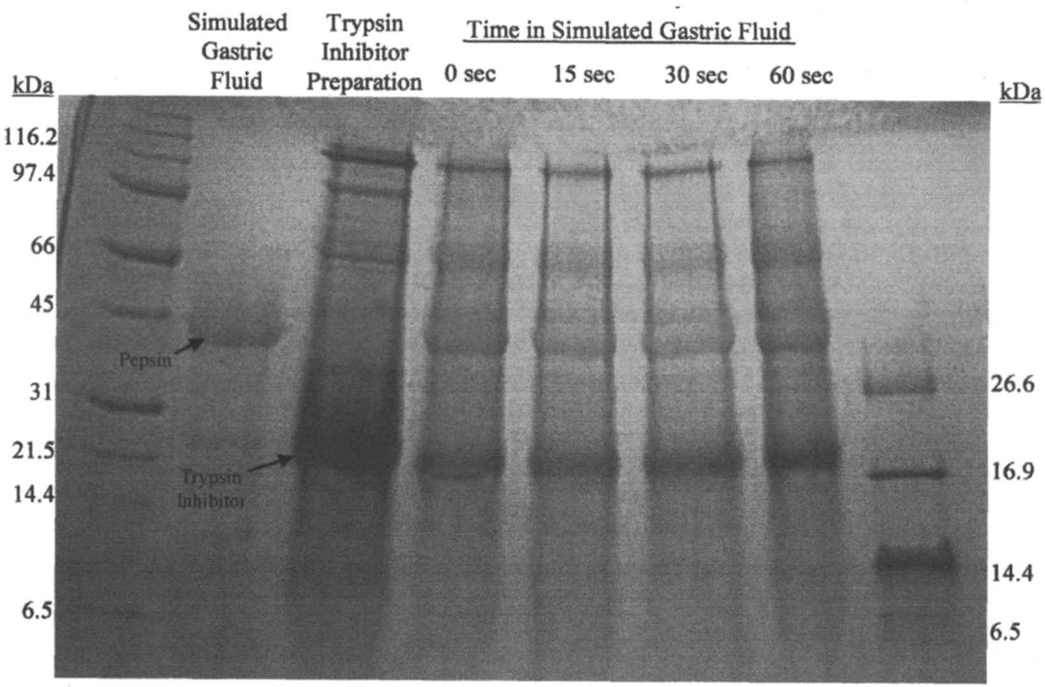
Simulated Gastric Fluid
Trypsin Inhibitor Preparation
Time in Simulated Gastric Fluid
0 sec
15 sec
30 sec
60 sec
kDa
116.2
97.4
66
45
31
21.5
14.4
6.5
Pepsin
Trypsin Inhibitor
kDa
26.6
16.9
14.4
6.5

Figure 1. SDS-PAGE results for soybean trypsin inhibitor in 0.32% pepsin solution.

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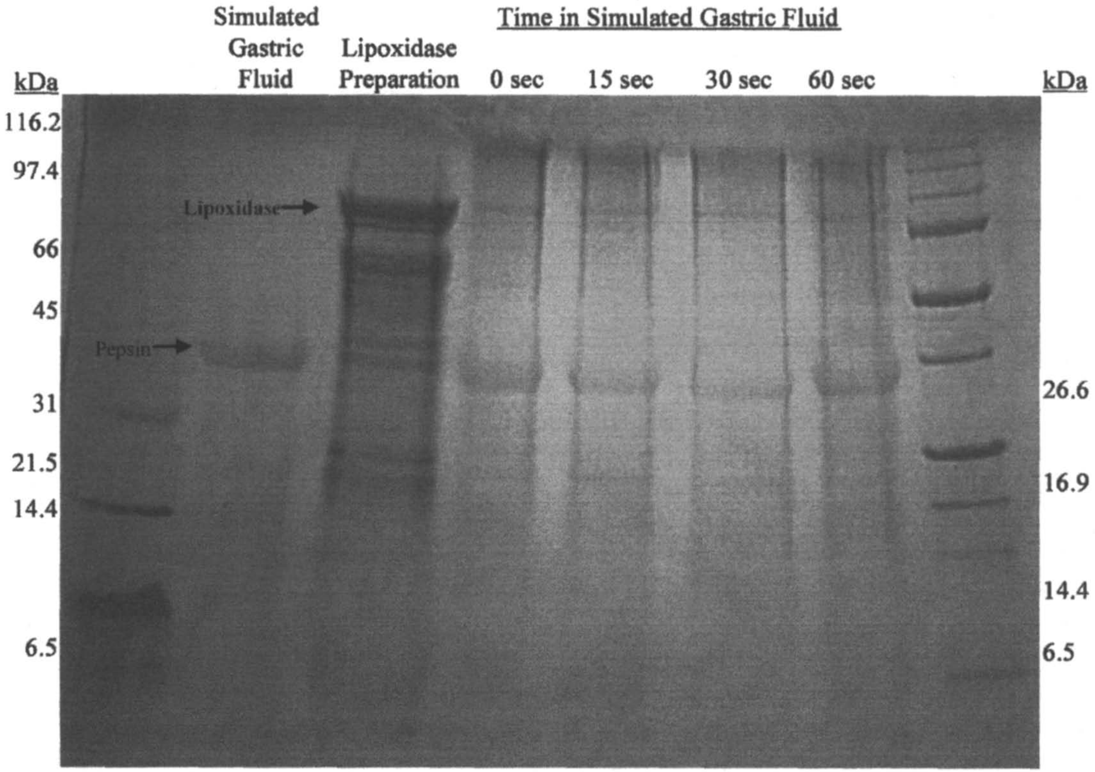
Simulated Gastric Fluid
Lipoxidase Preparation
Time in Simulated Gastric Fluid
0 sec
15 sec
30 sec
60 sec
kDa
116.2
97.4
66
45
31
21.5
14.4
6.5
Lipoxidase
Pepsin
kDa
26.6
16.9
14.4
6.5

Figure 2. SDS-PAGE results for soybean lipoxidase in 0.32% pepsin solution.

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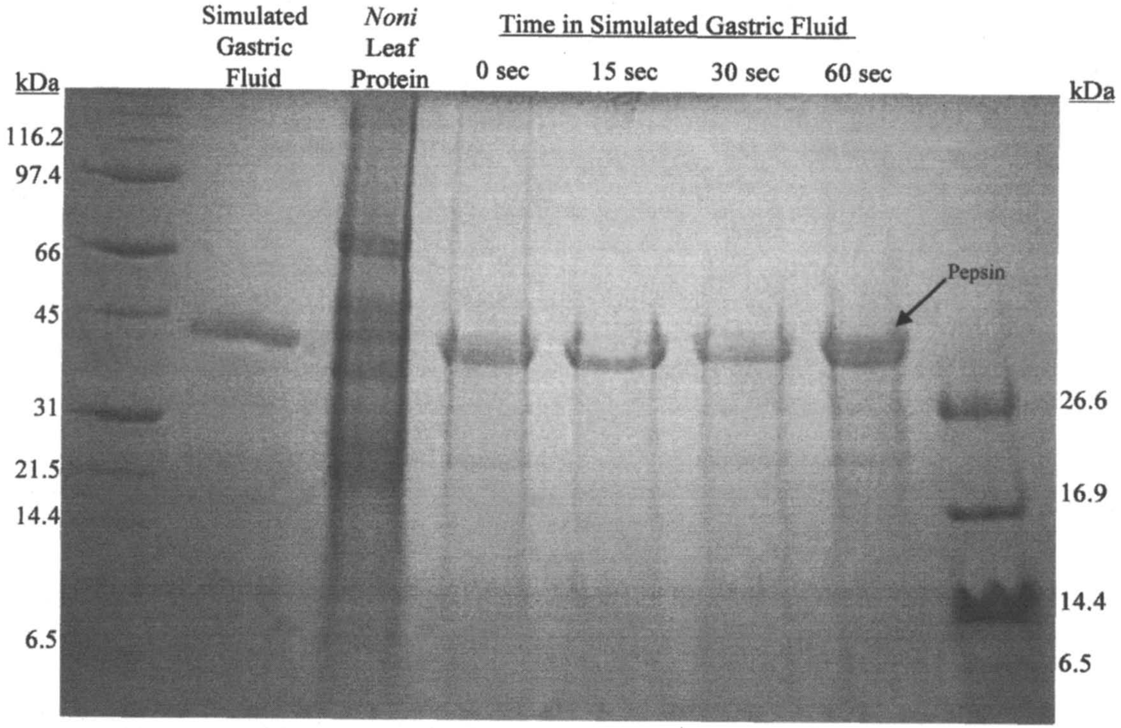
Simulated Gastric Fluid
Noni Leaf Protein
Time in Simulated Gastric Fluid
0 sec
15 sec
30 sec
60 sec
kDa
116.2
97.4
66
45
31
21.5
14.4
6.5
kDa
Pepsin
26.6
16.9
14.4
6.5

Figure 3. SDS-PAGE results for M. citrifolia *leaf protein in 0.32% pepsin solution.*

Conclusion

M. citrifolia belongs to the Rubiaceae family, which is not one of the botanical families that contain common allergenic food plants (*24*). Recorded human use and toxicity tests reveal that noni leaves are safe for consumption. Further, proteins in *M. citrifolia* leaf are readily digested by pepsin in vitro. Thus, these proteins will be easily digested when the leaf is eaten by humans.

As pepsin resistance is a typical characteristic of food allergens, a lack of resistance corresponds to a lack of allergenic potential. These findings corroborate the modified acute systemic anaphylaxis test results, indicating that *M. citrifolia* leaf poses little or no allergenic risk.

The apparent lack of toxicity and allergenicity, and the nutritive value of *M. citrifolia* leaf demonstrate that it is a healthy and viable ingredient in global food markets. It is also a food resource in the tropics, particularly in areas where conditions do not permit the sustained growth of other crop foods. We have observed that *M. citrifolia* is a hardy plant that grows in many soil types and requires very little, if any, care. These properties make it an ideal plant for further agricultural development.

This is the first reported use of the pepsin resistance test in the evaluation of a whole natural food, newly introduced from the tropics. Much attention has been focused on methods to evaluate novel proteins in genetically modified food crops, which methods are not very feasible when applied to natural plant foods. On the other hand, very little attention has been paid to developing possible methods for evaluating underutilized natural plant foods from underdeveloped nations. Pepsin resistance is a useful property that should be examined as an initial screen for potential allergenicity, when foods are introduced from one population or region to another.

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Chapter 27

Occupational Asthma and Rhinitis

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The workplace is emerging as an increasingly important venue for the development of rhinitis and asthma. There is no question that allergic diseases of the respiratory tract due to occupational exposure are on the increase. The clinician's diagnostic index of suspicion must be high so that a diagnosis of occupational rhinitis and/or asthma can be made in a timely fashion. Altering the environment of the workplace or removing the patient from that workplace may spare the patient permanent damage.

The workplace is becoming increasingly important to human health for a number of reasons. First, occupational allergic diseases are quite common and appear to be on the increase. The incidence of these diseases is undoubtedly underestimated, either because of a failure to diagnose or a reluctance on the part of workers during a difficult economy to complain for fear of losing their job. Secondly, there is increasing appreciation of the workplace as an arena for exposure to allergens. There has been an assumption that low molecular weight substances commonly encountered in the working environment were not of sufficient size to elicit an immunologic response. What we have come to learn is while many of these substances are not immunogenic in and of themselves, they are excellent haptens. Therefore they can combine with tissue proteins such as human serum albumin in the respiratory tract with the resultant hapten-protein conjugate serving as a potent immunogen. A third reason for a clinician to become increasingly concerned with the patient's workplace is that it provides an opportunity to practice preventive medicine. Diagnosing an occupational disease, recognizing the substance(s) that are responsible, and then altering the environment or removing the patient from that environment will benefit the patient greatly. This review will deal with occupational rhinitis and asthma.

Occupational Rhinitis

Occupational rhinitis has been defined as the episodic, work related occurrence of sneezing, nasal discharge, and nasal obstruction (*1*). This condition may have a profound effect on the worker, resulting in performance deficits, reduced productivity, and psychosocial problems. Occupational rhinitis often coexists with occupational asthma. Although it is commonly accepted that allergic rhinitis may precede asthma (*2*), reports of occupational rhinitis turning into asthma are few. In one report (*3*) from Finland that examined cases of occupational rhinitis and asthma reported to the Finnish Register of Occupational Diseases, most of the occupational asthma patients were older than those with rhinitis, suggesting that rhinitis precedes asthma.

The incidence of occupational rhinitis is not known, but in a survey of laboratory workers with allergic symptoms, 100% of those affected had rhinitis with conjunctivitis, whereas only 71% had asthma (*4*).

Causes of Occupational Rhinitis

The causes of occupational rhinitis may be placed in several classes (*5*). The first is annoyance, which occurs in individuals with a heightened olfactory awareness to such substances as perfumes and detergents. The likelihood of developing annoyance reactions is increased by nasal polyposis, sinusitis, tobacco abuse, and overuse of over-the-counter nasal decongestants or illicit drugs (*6*).

The second is irritational, meaning the nonspecific inflammation of the nose that has no immunologic or allergic basis. Exposure to substances, such as cigarette smoke, formalin, and capsaicin results in the release of substance P, a sensory transmitter that triggers a neurogenic inflammatory response. This type of occupational rhinitis is seen in patients who work in an enclosed environment and who are exposed to materials such as paints, talc, and coal dust.

A third category is corrosive, resulting from exposure to a high concentration of irritating and soluble chemical gases, such as chlorine or ammonia. This leads to such a degree of nasal inflammation that the mucosa burns and ulceration may occur, leading to permanent changes in physiologic functions of the olfactory system.

The final category is immunologic. By that we mean an allergic response mediated by IgE, with resultant early and late nasal reactions. High-molecular-weight allergens or low-molecular-weight allergens bound to proteins can elicit

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Table I. Causative Agents and Typical Exposures in Occupational Rhinitis

Cause	*Agent*	*Exposure*
Annoyance	Detergents	Supermarket
	Perfumes	Department store
	Cooking	Kitchen
Irritational	Air pollutants	Outdoor or inner-city workplace
	Cigarette smoke	Any workplace
	Cold air	Meat packer
	Hairspray	Department store
	Talc	Cosmetics industry
Immunologic	Natural guar gum	Carpet worker
	Latex	Nurse
	Psyllium	Nursing home
	Rats	Laboratory researcher
	Synthetic acid anhydride	Epoxy worker
	Platinum	Jeweler
	Toluene diisocyanate	Spray painter

an allergic response. Some causative agents and typical exposures resulting in occupational rhinitis are summarized in Table I.

Psyllium, a major component of popular bulk laxatives, may cause rhinitis in persons who work in laxative-producing factories or in nurses who dispense the medication (*7*). Guar gum commonly is reported as a cause of occupational rhinitis and asthma, especially in Europe. This gelling and thickening agent affects workers in the food industry. Carpet workers may become sensitive to guar gum through its use as a fixator of dye to fiber and as an insulator in rubber cables (*8*).

Laboratory workers are commonly affected with occupational rhinitis and asthma with more than 30% of these workers becoming sensitized. The rat is the most commonly implicated animal, and, as stated earlier, rhinitis with conjunctivitis is the most common complaint among these workers (*4*).

In one of the earliest reports of sensitivity to western red cedar dust (plicatic acid), approximately half of the affected workers had associated rhinitis (*9*). In another report, rhinitis caused by exposure to western red cedar preceded the onset of asthma (*10*).

Latex-induced occupational rhinitis is increasingly diagnosed. OSHA estimates that 8% to 12% of healthcare workers are sensitized to latex (*11*). In one report, a nurse experienced immediate itching, redness, and irritation of the hands after direct skin contact with natural rubber latex gloves over a 6-year period (*12*). With use of vinyl gloves, her symptoms improved. Coworkers, however, still used latex gloves, and the nurse began noticing symptoms of rhinitis at work because of the significant amount of airborne natural rubber latex antigen. Nasal provocation and skin test to latex were positive, and she had elevated serum levels of latex-specific IgE.

Diisocyanates are highly reactive chemicals that are encountered by foundry workers, polyurethane foam makers, and spray painters. It is the most common class of reagents that cause occupational asthma. Diisocyanates may cause a nonspecific irritant rhinitis, and in a small number of workers who have an IgE sensitivity to diisocyanates, allergic rhinitis may result. The risk for occupational rhinitis or occupational asthma is related directly to the exposure level; atopy and smoking do not seem to be major risk factors (*6*).

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Diagnosis of Occupational Rhinitis

One should consider the following steps in making the diagnosis of occupational rhinitis: history and physical examination, a site visit, determination of IgE by either skin tests or in vitro tests, and nasal challenge.

History and Physical Examination

The association of nasal congestion, sneezing, and increased nasal secretions with exposure to the workplace provides the first clue to the diagnosis. Occupational rhinitis may be associated with an isolated late reaction; therefore, symptoms might not appear until several hours after leaving the workplace.

The primary symptom of a late reaction is nasal congestion rather than sneezing, itching, or rhinorrhea. Symptoms usually abate when the patient is away from the workplace on weekends or holidays, but chronic exposure may require several days away from work before the symptoms remit. Physical examination during the time of active rhinitis reveals swollen nasal turbinates and evidence of increased secretions. Nasal findings may be minimal in patients with annoyance causes of occupational rhinitis, whereas corrosive causes reveal ulcerated and hemorrhagic mucosa.

Infectious paranasal sinusitis should be excluded, because it is such a common occurrence. Occupational rhinitis may predispose to sinusitis. The mechanism is believed to be inflammatory swelling of the nasal mucosa, which leads to blockage of the ostia and decreased aeration and drainage from the sinuses. Symptoms of sinusitis include persistent nasal blockage and purulent postnasal drainage.

Site Visit

A site visit to the workplace is frequently helpful in terms of eliciting the exact cause of occupational rhinitis. The patient may be unaware of the ingredients to which he or she is exposed and may forget certain routine acts in relating the occupational history. A site visit enables the physician to see the patient in action and to obtain a more accurate picture of the types of exposure. Material Safety Data Sheets (MSDSs) must be available by law at the workplace. These data give detailed information about the substances to which the worker is exposed and the health effects of each substance.

Determination of IgE

If the occupational rhinitis is caused by an IgE mechanism, appropriate skin tests or *in vitro* radioimmunoassay testing may be performed. Skin testing is useful in allergic occupational rhinitis if the disease is caused by animal, dust, latex, or mold exposure. Low molecular weight substances may need to be conjugated with serum albumin to elicit a response.

Nasal Challenge

Direct nasal challenge can be performed naturally at the work place or under controlled circumstances in the office or laboratory. Responses may be quantified by symptom score or rhinomanometry. A simple technique that is used widely in Europe is to impregnate a piece of cotton wool with the offending agent, such as guar gum, and to apply this wool directly to the nasal mucosa (*8*). An aqueous solution also can be blown into the nose from a syringe. One hour later, the nose is examined, and the patient's symptoms are noted. Nasal challenge is performed best when the patient is not taking antihistamines and when symptoms are minimal. The technique of rhinomanometry, which measures changes in nasal airway resistance, is the preferred technique to objectively measure the response to nasal challenge (*13*). It has been suggested that skin prick tests combined with specific IgE tests for latex are highly specific and sensitive for latex allergy and that nasal provocation should be performed only in symptomatic glove users who have borderline results of skin and humoral tests for latex (*14*).

Nasal challenge can provide insights into the physical characteristics of materials that may cause occupational rhinitis. Blainey *et al.* (*10*) reported the case of a carpenter whose job involved using a circular saw and planing wood. The carpenter noted that his nose became blocked within 15 minutes of cutting western red cedar. Nasal challenge with coarse dust derived from sawing the

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cedar wood resulted in no response, but challenge with the fine dust that was obtained from planing or sanding the wood caused a significant isolated late increase in nasal airway resistance (6.5 hours after the challenge began). Acoustic rhinometry, a simple, easily reproducible, and less invasive procedure has been reported to be useful in diagnosing occupational rhinitis (*15*).

Disease Prevention

Occupational rhinitis affords the physician an opportunity to practice preventive medicine, the most definitive approach to managing disease processes. One frequently suggested method is to screen applicants for jobs in which there is a possibility of workplace sensitization. By means of history and skin tests, it is possible to isolate potential workers who are basically atopic.

In a study performed on laboratory workers, screening applicants in such a manner proved to be an inefficient method of reducing the incidence of allergy to laboratory animals. To exclude 12 workers who developed asthma and rhinitis, 71 workers with positive skin test results would have to be rejected. If the 41 workers with positive skin test results and a positive allergic history were rejected, only 44% of those who went on to develop asthma and rhinitis caused by rats and only 23% those who only developed rhinitis would be rejected. In summary, a significant number of individuals with rat allergies were not initially atopic, and most atopic individuals did not become sensitive to rats (*16*).

A second approach to preventive medicine is to institute environmental control measures, such as providing adequate ventilation. The offending allergen and its source should be recognized. Laboratory workers who become sensitive to rats largely react to rat urinary protein rather than epithelium. It may be possible for the sensitive worker to perform surgery on rats if the cage containing the rats, which usually is soaked with urine, is kept out of the laboratory. Remarkable results can be obtained by placing high-efficiency particulate air filters in areas of the laboratory where rat exposure is high.

Pharmacologic Management

Pharmacotherapy of occupational rhinitis is similar to that of other types of rhinitis (*17*). Antihistamines are a mainstay of managing allergic and non-allergic rhinitis. When the patient's symptoms are frequent or unpredictable, regular use of an over-the-counter or prescription antihistamine may be necessary. When symptoms are episodic and predictable, prophylactic use of antihistamines before exposure to irritants or allergens is generally sufficient.

The choice of the agent often is based on trial and error, according to frequency of side effects and efficacy. The sedative side effects of conventional

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antihistamines may be more bothersome than the rhinitis itself, especially when the goal of treatment is to increase alertness and productivity.

The second-generation non-sedating antihistamines loratadine (Claritin), fexofenadine (Allegra), and ceterizine (Zyrtec) are better for treating patients who need to be awake and alert at work. The duration of treatment is variable. Because antihistamines are only palliative, they should be continued until the patient can control symptoms by avoiding exposure to the offending agent.

Topical agents are effective for managing rhinoconjunctivitis. Ophthalmic solutions of antihistamines, decongestants, mast cell stabilizers, and nonsteroidal anti-inflammatory agents provide effective, as-needed prophylaxis or symptomatic treatment of rhinoconjunctivitis. Saline nasal spray is useful for non-allergic rhinitis. The addition of anticholinergic agents, such as ipratropium bromide, benefits patients who have anterior or posterior drainage. Topical glucocorticoid nasal sprays are effective for allergic and non-allergic rhinitis. They often need to be taken on a regular daily schedule but can be effective on an as-needed basis. The same rule applies to antihistamine nasal sprays.

Allergen immunotherapy generally is not recommended for patients with occupational rhinitis. Immunotherapy has no role in the management of rhinitis that is caused by irritative substances, and its value in alleviating allergic occupational rhinitis is limited by the unavailability of occupational antigens.

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Occupational Asthma (OA)

Many papers and several excellent text books have been written about occupational asthma (*18, 19*). The subject has been covered quite extensively, particularly in the textbooks. Therefore, I would like to summarize some major points about occupational asthma.

Mechanisms

OA can be placed into four categories based on underlying mechanisms.

The first is reflex bronchoconstriction which appears to be due to a neurogenic reflex resulting from insult to the bronchial epithelium. The damage that occurs leads to marked inflammation with resultant fibrosis, increased bronchial smooth muscle tone, and mucous hypersecretion. The best example of this is reactive airways dysfunction syndrome (RADS) (*20*). Agents implicated in this entity are ammonia, fire smoke, chlorine gas, HCl, and H_2S. The onset of symptoms including cough and shortness of breath occurs soon after exposure, usually as a result of an accidental spill. Airway reactivity may persist after recovery from the acute episode.

A second category of OA is due to allergy. There are a number of characteristics that would prompt a clinician to believe that a particular case of OA was allergic or immunologic in nature. These include a latency or sensitization period with asthma appearing after repeated exposure, its occurrence in only a relatively small proportion of the exposed workers, an increase in sensitivity with time, eliciting of reactions by amounts less than is encountered at the workplace, and evidence of immunologic memory or anamnesis. After removal from the workplace for a period of time, reintroduction results in symptoms in a much shorter time than after the original exposure.

Some common causes of allergic OA are shown in Table II along with the occupational exposure.

Table II. Some Causes of IgE Mediated Occupational Asthma

Agent	*Occupation*
trypsin	plastic workers
animal dander	researchers
grain dust	farmers
platinum	jewelers
flour	bakers
psyllium	nurses
papain	lens makers
trimellitic anhydride	spray painters

Determinants of OA include both environmental and host factors. The concentration and duration of exposure to the offending substance is quite important in the production of OA. The effect of smoking is variable but this may interact with atopy or underlying allergy in sensitization to higher molecular weight (MW) substances. A background of atopy appears to be a risk factor for sensitization to high MW substances but the predictive value is low.

Most cases of OA begin within one to two years of exposure with sensitization to lower MW substances shorter than to high MW substances.

A number of low MW agents may cause OA on a nonallergic basis. One of the most common is toluene diisocyanate (TDI) encountered in automobile assembly plants and paint and adhesives factories. Exposure to TDI causes sensitization and subsequent respiratory symptoms (*21*). There is no clear evidence of an IgE mediated reaction .

A final category of OA is due to an ingredient in the cotton bract that is thought to result in nonimmunologic release of histamine from mast cells leading to byssinosis. At risk of developing this disease are cotton, flax and soft

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hemp workers. Through appropriate environmental measures in the work place, the incidence of byssinoses has markedly declined.

Diagnosis of OA

The first step in diagnosing OA is to determine if the patient indeed has asthma. The presence of existing asthma is confirmed by spirometry or by establishing airway hyperresponsiveness to methacholine. The next step is to establish that an agent in the workplace is causing asthma. The history will give an important clue if asthma worsens during the work week and improves on weekends and holidays. This may be misleading, however, in that, as will be discussed later, the patient may have an isolated late response with symptoms appearing hours after leaving the work place.

If the occupational asthma is due to an IgE mediated reaction, skin tests or serologic tests can verify the presence of IgE antibody.

Studying the response of the patient to a "natural" challenge consists of monitoring symptoms and peak expiratory flow rate before and after a work shift. If positive, then the association is made. If negative, peak expiratory flow should be measured three times a day for three weeks. At some time, nonspecific bronchial reactivity can be measured before and after work.

A controlled bronchial challenge can be performed in certain specialized centers. The challenge may be performed to dusts, aerosols, vapors, and gases. Such challenges have demonstrated three distinct types of responses; an immediate response with forced expiratory volume in 1 second (FEV1) declining in a few minutes, a dual response in which the immediate fall in FEV1 is followed by another generally greater fall in FEV1 4 to 6 hours later and an isolated late response in which the FEV1 does not fall until four to six hours have passed. Interestingly, an isolated late response appears to be more commonly seen in occupational asthma than in asthma due to common environmental allergens (*22*).

Prognosis

A number of factors affect the prognosis of occupational asthma. Particularly important is the duration of symptoms before the diagnosis is made. Also important are the severity and frequency of attacks. The worse the lung function, the worse the prognosis. Finally, a dual bronchial response to a challenge and a higher degree of bronchial hyperreactivity both contribute to a worse prognosis. Most patients will experience symptomatic improvement after leaving the workplace, especially if the diagnosis is established in a timely manner and the patient is promptly removed. While there is suggestive evidence

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that bronchial responsiveness may be acquired rather than a predisposing factor in occupational asthma, more well controlled prospective studies are needed (*24*).

Therapy

As was discussed in the section on occupational rhinitis, environmental control is vitally important in managing occupational asthma. The physician must truly serve as the patient's advocate. Every effort should be made to alter the working environment to the benefit of the patient. These would include the control measures referred to previously. Failing that, the patient should be reassigned to a different working environment. The same agents useful for treatment of other types of asthma are certainly appropriate for the patient with occupational asthma.

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Chapter 28

Mitigation of Soybean Allergy by Development of Low Allergen Content Seeds

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With the increasing incidence of food allergy in the industrialized world, public awareness and concern has prompted intensified efforts to treat and mitigate allergic reactions. Much of the effort has been directed at avoidance by better labeling and awareness in conjunction with various forms of medical intervention. An alternative approach is to alter the allergen content of the food which is essentially a variation of avoidance, avoiding the allergen not the food. Soybean is one of the "big eight" food allergens difficult to avoid because of its wide use in processed food. Eliminating most of the allergens identified by IgE cross-reactivity appears feasible. This presents the potential to produce low allergen variants of otherwise significantly allergenic foods. Discussed here is the process of producing low allergenic soybeans and the implications that may result from their testing and use.

In 2006 an important piece of legislation, The Food Allergen Labeling and Consumer Protection Act (FALCPA), became law, mandating plain language labeling of processed foods to identify eight major allergens. For many of these allergens such as peanut, tree nuts, wheat, and dairy the potential adverse health consequences of consumption are well known and in the knowledge base of the general public. Among the eight allergens mandated to be labeled is soybean. Although soybean is generally considered to be an allergen of widespread importance, unlike many other food allergens it has been hidden both as a food constituent often cryptically labeled as vegetable protein or similar term and in

U.S. government work. Published 2008 American Chemical Society.

the sense that its allergenicity is not widely known to the general public (*1*, for review). In addition to its impact as a human allergen soybean has significance as an allergen to a wide variety of production and companion animals (*1*, for review). In the United States and many other countries with consumption of processed foods and feed, the soybean component is large and because soybean protein is both relatively well balanced in composition and inexpensive its inclusion in food and feed is growing. Using soybean proteins has been shown to be so advantageous for the world food and feed supply that its effect as a food allergen is something that needs to be managed. Because soybean food and feed allegenicity affects such a broad range of consumers, managing the allergic impact by medical intervention may not prove to be economically feasible, especially for feed uses. Because of the need to mitigate soybean allergy and limits of cost factors in its mitigation, the management of soybean allergenicity needs to include paradigms outside the high cost of medical intervention. For this reason soybean is an important model to test other approaches, specifically the concept of agricultural approaches: using plant improvement to address and mitigate soybean food and feed allergy. This chapter describes the progress, opportunities, and down-stream impact of using modern agricultural approaches.

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Soybean Allergy

Wheat, dairy, eggs, and soybean are often cited as major sources of food allergies. Avoidance of these foods is difficult because they are widely used in prepared foods and other products (for example, *2-4*). Soybean allergies are manifested as both respiratory and systemic symptoms. The respiratory allergy caused by dust is largely occupational, although interestingly this allergy also manifests from exposure to beanbag chairs (*5*). The allergies to consumption occur not only from foods, but also from exposure to other soy products, such as cosmetics (*6*). The widespread use of soy-based formula, especially when children are sensitive to milk, exposes a large fraction of babies to soybean allergens and increases the potential for soybean sensitivity (*7*, *8*). Soybean allergies are also common among farm animals, including salmon, trout (*9*, *10*), and young cattle (*11*), with neonatal pigs among those most affected (*12-18*). Soybean protein is used in thousands of processed foods throughout the industrialized world and is a staple in Asia. Soy milk and dairy product replacement is growing in acceptance not only by people sensitive to lactose and/or milk proteins, but also for health considerations. Soybean allergy occurs with a far higher frequency in infants and children (*19*, *20*). Soybean sensitivity is estimated to occur in 5-8% of children and 1-2% of adult humans; the allergic reaction is only rarely life-threatening, with the primary adverse reactions to consumption being atopic (skin) reactions and gastric distress. But with the wide use of soybean in processed food, the allergy to soybean takes on a large and significant impact in the lives of millions of people. Soybean allergy is often synergistic with other food allergies. In infants exhibiting milk allergy to milk-based formula, 25-50% will also acquire sensitivity to soybean-based formula (*21*, *22*). Soybean is a member of the legume family that includes many other allergenic seeds including peanut, lentil, chickpea, and pea. The allergenic seed proteins of the legumes are closely homologous and have high sequence identity. There are many documented patient histories where the development of an allergenic response to one legume leads to development of allergy to another. One of the more common patterns is initial sensitivity to peanut and the subsequent development of soy allergy

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Soybean Allergens

Soybean allergens consist of a diverse set of seed proteins for soy consumption allergy (*23*, *24*) and for baker's asthma (*25-27*) and of a few seed coat proteins for the occupational allergy also referred to as Barcelona syndrome. Soy allergens are not unique to soy; all of the significant allergens have allergenic and for the most part non-allergenic homologues in other plants. Soybean seed composition is due to conservation of proteins quite similar to other seeds, especially members of the legume family such as peanut, pea, and green beans. Seeds have evolved to maximize storage of reserve substances needed to support post-germination growth - storage proteins to provide carbon, sulfur, and nitrogen reserves, and oil and carbohydrate, both oligosaccharides and starch, as carbon reserves (*28*, for review). Seeds mature in a highly determinant developmental pattern, with differentiation to a fixed number of cells occurring early in development and the remaining growth cellular enlargement occurring in parallel to the deposition of the reserve substances. Because seeds are a highly nutritious concentrated package, they are a highly desirable food source for animals (from mammals to insects) and for fungi and bacteria. It is the concentrated nutritional advantage coupled with the near dry stable state for storage, trade, and shipping that have made seed crops the heart of agriculture worldwide. Seeds have evolved defense mechanisms to limit the ability of animal consumption as well as fungal and bacterial growth. Among the defense mechanisms present in soybeans and other seeds are inhibitors of key digestive hydrolases, including protease and amylases, lectins that bind to the surfaces of cells impairing physiology especially in the digestive tract, and other possible defense proteins, including a protein that binds a secreted compound from *Pseudomonas* as major seed-transmitted soybean pathogen (*29*). Most soy allergens are localized in the soy protein storage vacuole, an organelle that has dual functions as an intracellular protein storage site and digestion site of the storage protein and other cellular constituents, making the organelle functionally equivalent to the animal lysosome. The defense function of soy proteins is a key consideration in soy allergen and generally for other seeds as well. The physiological activity of some seed proteins against animals and microbes to impede potential consumption, coupled with a general resistance against digestive enzymes, results in seed proteins being very stable even in conditions that would easily result in hydrolysis. The consequence of this is that seed proteins tend to score highly as potential allergenic risks in models such as the WHO Allergenic-Risk Assessment Tree.

Soybean proteins identified as binding IgEs from consumption by soy-sensitive individuals (*30*) include both 7S vicilin-type (conglycinin) (*31*) and 11S legumin-type (glycinin) (*32-34*) storage proteins, soybean agglutinin, Kunitz trypsin inhibitor, 2S albumin, lipoxygenase, P34 (which is a member of the papain superfamily of proteases) (*35-37*), profilin, and a few other proteins including one with homology to chlorophyll binding protein and another related to pathogen defense proteins. In addition to the consumption allergens, airborne soy-dust allergens from soybean hulls induce occupational allergic reactions that are cause by low molecular weight hydrophobic proteins with homology to lipid transfer proteins. All of the proteins that are identified by immunological assay from sensitive patient sera have homologues in other plants that are also allergens. Many of these proteins - the storage proteins, agglutinins, and protease inhibitors - are significant allergens in diverse plants and because there is considerable sequence conservation in all of these proteins, the development of sensitization to one specie's protein can spread to other closely related sequences and new species, continually broadening the range of sensitization to more foods. The clinical manifestation of the phenomena is that patients will exhibit an increasingly broader range of food sensitivity with its consequent impact on lifestyle.

Most of the epitopes described for soy allergens are linear (*32-36*). Food processing adversely affects allergenicity of peanut proteins in roasted peanuts. Whether similar effects occur in soybean, which is processed and used in a wide variety of ways, remains to be investigated with the implication of how processing affects allergenic epitopes.

The Immunodominant Soybean Allergen P34/Gly m Bd 30k

P34, a soybean seed protein (*38*), was recognized as a major soybean allergen by Ogawa *et al.* (*37*), who surveyed proteins that bound IgEs derived from soybean sensitive people. The P34 protein was termed Gly m Bd 30k in accordance with nomenclature used for allergens. The human allergenicity of P34/Gly m Bd 30k is demonstrated in the adjacent sodium dodecyl sulfate

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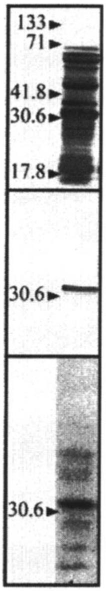

polyacrylamide gel electrophoresis (SDS/PAGE) and immunoblot. The upper panel shows a gel of the total protein from a mature soybean seed extract. The middle panel shows the cross-reactivity with an anti-P34 monoclonal antibody resulting in the labeling of a single 34 kDa polypeptide. The bottom panel shows the consequences of probing a replicate blot with a sera pool obtained from soybean-sensitive infants and labeling the bound IgE antibodies with a second antibody (*39*). Note that the immunodominant protein indicated by IgE binding is the 34 kDa P34/Gly m Bd 30k.

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P34/Gly m Bd 30k is a Non-proteolytic Papain Superfamily Member from Legumes that may have a Pathogen Defense Function

P34/ Gly m Bd 30k is a member of the papain superfamily of cysteine proteases (*40*). It is a minor protein constituent of the protein storage vacuoles of soybean, co-localized with the storage proteins and other proteins including allergens such as Kunitz trypsin inhibitor and soybean agglutinin. P34 is produced during mid to late seed maturation in the embryonic cells of the soybean seed and accumulates to about 1% of the total seed protein. Like other members of the family it possess a large prodomain that has been shown in other members to lie in the active site groove acting as an inhibitor until the protein is activated (see diagram below for protein structure). P34 possesses three disulfide bridges and other structural features highly conserved in animal, plant, and fungal examples of this family. What separates P34/Gy m Bd 30k from all other members of this family is the substitution of glycine for the catalytic cysteine, rendering the protein apparently proteolytically inactive. The primary translation product of the gene encoding P34/Gly m Bd 30k is a pre-proglycoprotein that undergoes co-translational removal of a signal sequence and high mannose glycosylation of the prodomain (*41*).

Most papain superfamily members self-activate under acidic reducing conditions, which occur when the pro-protein progresses from the Golgi to either a lytic compartment (vacuole, lysosome) or the extracellular space. In contrast, processing of P34/Gly m Bd 30k removes the pro-domain with cleavage occurring at the C-terminal of Asn122 (*41*). In seeds, the Asn-specific cleavage is characteristic of post-translational processing of protein storage vacuole proteins, including the 11S storage proteins. The initial processing to the mature 34 kDa form occurs during seed maturation. Another processing step during seed germination, results in the loss of ten amino acids (123-132) from the N-terminus and a 32 kDa protein that appears to be a quantitative conversion at 3 days of

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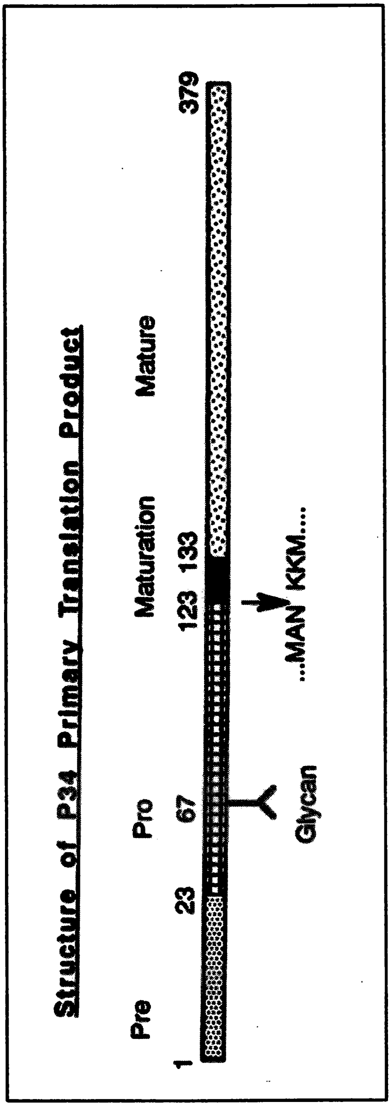

Structure of P34 Primary Translation Product

Pre
Pro
Maturation
Mature
1
23
67
123
133
379
Glycan
...MAN KKM...

seedling growth. This occurs simultaneously with the onset of storage protein mobilization, although we have no data yet to support a role for P34 in storage protein mobilization. The removal of the N-terminal segment is novel among cysteine proteases, but its role in P34/Gly m Bd 30k function remains to be established.

There is some data that indicates that P34/Gly m Bd 30k may be a pathogen resistance or defense protein of soybean (*29*). These results showed that P34/Gly m Bd 30k is also produced in vegetative cells, although at a much lower level, and that its presence may be related to a pathogen response, possibly to *Pseudomonas* infection, with the protein's gene mapping the RPG4 *Pseudomonas* resistance locus. P34/Gly m Bd 30k binds a syringolipid elicitor from *Pseudomonas* with the protein purified by affinity chromatography from leaf extracts. So there is a correlation with both the locus for resistance and a specific interaction with a *Pseudomonas* elicitor. Although P34/Gly m Bd 30k appeared to be a unique example of the papain superfamily with its Gly substitution for the catalytic cysteine, there are accessions in Genbank for the legume Jicama with a very similar protein including the catalytic residue substitution. This indicates that soybean P34/Gly m Bd 30k is the first example of a group of non-proteolytic papain superfamily members found broadly in new and old world legumes. This result is a caution of a potential long-range problem for creation of allergen-free crops. A large proportion of allergenic proteins are also defense proteins, so the elimination of the allergens may adversely alter agronomic potential. As new lines are developed lacking one or more allergens, this will need to be carefully monitored using tests, particularly a field test, to examine pathogen resistance.

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P34/Gly m Bd 30k is not Unique as a Cysteine Protease Human Allergen

Many allergenic proteins are members of the cysteine protease enzyme superfamily. For example, the dust mite fecal allergen Der1p is a well characterized cysteine protease (see *42*, *43* for examples). Actinidin, the primary cysteine protease of kiwi fruit, is a food allergen (*44*). Other cysteine proteases from plants induce occupational and food allergies because of the use of these enzymes to treat foodstuffs (see *45, 46* for examples). Allergies to papain, the archetype cysteine protease (*46, 47*), occur by exposure to contact lens solution, where it is used to strip proteins from the lens (*48*), from exposure to fruit juices in throat lozenges (*49*), and from its use as a meat tenderizer (*50*).

One model for allergenicity of the papain superfamily is that the destructive proteolytic activity has a major role in inducing the immune response to these proteins (*43*, *46*, *51*), however, this model would not apply to P34 with its lack of protease activity. More information is required to understand why some papain superfamily members are significant allergens and what is the relationship between the allergenic and non-allergenic varieties. Crystal structures have been determined for papain, actinidin, and Der 1p, but immunogenic epitope data is only available for Der 1p (*43*). A crystal structure of P34/Gly m Bd 30k is in progess that will facilitate comparisons with known epitopes and with other cysteine protease allergens. Combined with the already elucidated IgE epitopes, this information will allow us to ask immunological questions on why P34/Gly m Bd 30k is a significant allergen, especially since it lacks the signature characteristics of being an active cysteine protease.

The production of experimental antibody reagents in mice and rabbits established that P34/Gly m Bd 30k is a "hot" antigen that elicits a strong response (unpublished results). These reagents are available for the current proposed project. P34/Gly m Bd 30k is also the strongest antigen IgE cross-reactivity in soy-sensitive infants. The human IgE epitopes of P34 from soy-sensitive people were determined by solid phase immunoassay using offset synthetic peptides corresponding to the entire open reading frame of the protein. These assays showed that 14 epitopes are distributed across the mature P34 sequence. The individual epitopes vary in intensity with some epitopes much "hotter" than others. The same procedure was used to examine immunological differences among unrelated soy-sensitive people with the assay of four unrelated people (*35*). These experiments showed that although P34 was an immunodominant allergen, the individual IgE epitopes varied in intensity among these subjects with a subset of the epitopes being the strongest in the majority of the subjects (*35*). The immunodominant epitopes were further examined by producing glycine mutations within the epitope to map the critical amino acids (*36*). Among six epitopes examined by this procedure, antigenicity was abolished by glycine substitution in any position of one epitope while in others there was only a single critical amino acid. Obtaining the detailed human immunology of P34 (*35*, *36*) was essential to establish background data to support the use of biotechnology to suppress P34 (*52*). For most food allergens, the linear epitopes consist of non-contiguous sequences that map to the surface of the allergenic proteins; however, for many large gene families there are highly allergenic examples while other family members exhibit little or no allergenicity. Papain superfamily cysteine proteases are among those proteins that have broad variations in allergenicity among family members. Thus, sequence relationships offer little insight to the prediction of allergenicity. Additional information is needed about the allergenic protein family members, including the three-dimensional structure of the protein and how it is perceived by the immune system.

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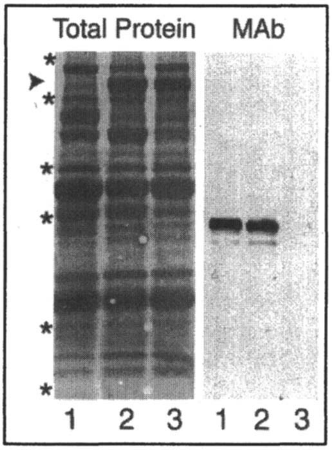
Mitigation of Soybean Allergies by Modifying Seed Protein Content

Biotechnology



There are several approaches that might produce a low allergen soybean, including suppressing expression of the allergen by biotechnology or discovery of a null variant from collections or by induced mutation. Using gene silencing techniques transgenic soybeans lacking P34/Gly m Bd 30k (*52*) have been produced. Suppression of P34/Gly m Bd 30k resulted in essentially eliminating all of the P34 protein's accumulation in resultant transgenic soybean seed, shown in the adjacent gels and blot. Lanes 1 and 2 are transgenic and conventional controls. Lane 3 demonstrates the effect of a P34/Gly m Bd 30k transgenic suppressor. This suppression appears to have no significant collateral consequences, since proteomic analysis of the seeds showed no unintended protein composition changes and no effect on growth, development or yield of the soybean plants. Suppressing P34/Gly m Bd 30k in soybeans was a first step and a demonstration in addressing the growing concerns of food allergies and their relationship to the development of genetically engineered crops.

Similar suppressions could be accomplished for other major soybean allergens including lectins, trypsin inhibitors, and storage proteins. For instance, the suppression of one of the major soybean storage proteins has been published and the resulting seeds are fully viable. Technically there is little to impede using gene suppression technology to remove one or more allergens from the same transgenic soybean line. Even with this success, the use of this technology to produce non-allergenic products is impeded by concerns about biotechnology and liability. So although directed transgenic modification is the best choice for reducing allergens from a technical standpoint, it is at present limited in acceptance and therefore suitability; so it remains an approach with a lot of future potential but many details to be resolved.

Conventional Approaches

The alternative to biotechnology would be to find a conventional variant lacking P34/Gly m Bd 30k and other allergens. This is an attractive alternative

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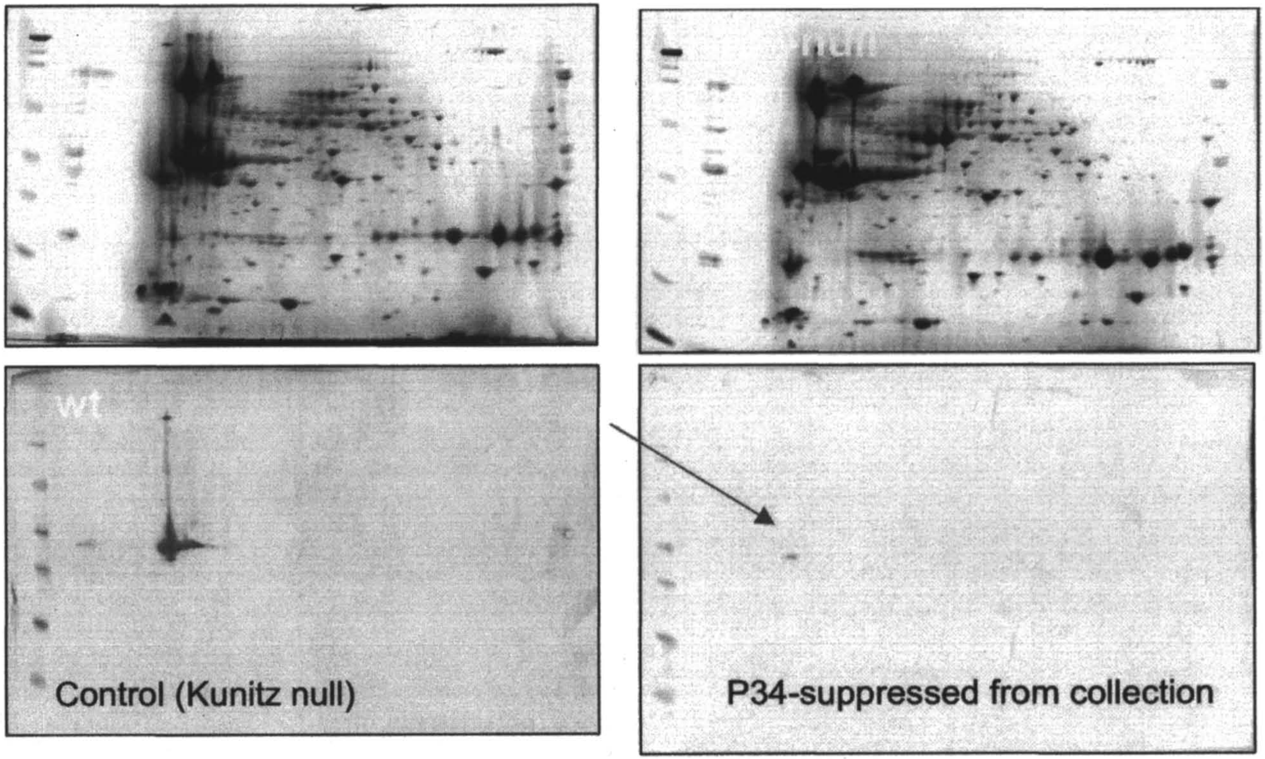
null
wt
Control (Kunitz null)
P34-suppressed from collection

Two-dimensional gels of a control soybean and one of the two P34/Gly m Bd 30k nulls isolated from the USDA soybean collection is shown. The upper panels show total stained protein while the lower panel shows immunoblots labeled with a P34/Gly m Bd 30k Mab.

for industry with concerns about regulatory issues of acceptance of transgenics. Conventional variants are not regulated, however claims of hyperallergenicity would be regulated. Cultivars lacking the allergens could be identified in germplasm collections and then crossed into elite germplasm. Ideally, an allergen null found in a collection should be a complete null in which the entire protein is non-functional or completly eliminated, as opposed to an epitope-specific null which would still likely cause allergic reactions in individuals sensitive to the epitopes still remaining.Initial surveys by the PI was unsuccessful in identifying a P34 null in soybean varieties (*39*). Since P34/Gly m Bd 30k contains 14 contiguous and noncontiguous linear epitopes (*35*), the likelihood of isolating a naturally occurring variant with a sufficient number of alterations to disrupt allergenicity while retaining the protein is extremely small. The other approach is to search for nulls among the germplasm collection, which is then functionally equivalent to the biotechnology approach of producing a null. There is an important difference in screening collections; the vast majority of the USDA National Collection of about 20,000 accessions are not anywhere close to be elite germplasm needed for agriculture. This is a collection over 100 years in the making containing all sorts of collected material. To make commercial use of any selected accession will require many years of breeding to cross that trait into elite germplasm. This is in marked contrast to biotechnology approaches where the transformation platforms are designed for straightforward transfer of traits into elite germplasm reducing the period needed to exhibit the trait in commercially viable material. Null lines for minor soybean allergens Kunitz Trypsin Inhibitor and Soybean Agglutinin have been identified. Recently the USDA soybean collection was screened for nulls of the immunodomnant soybean allergen P34/Gly m Bd 30k. Two closely related or identical accessions were isolated from the collection that lack the P34/Gly m Bd 30k protein (*53*).

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Issues for Acceptance of Low Allergen Variants of Allergenic Crops

There are few or no technical problems precluding the production and breeding of soybeans which lack enough allergens to constitute a large majority of the immunological binding capacity of the known allergenic proteins. This appears to be achievable in the near future. On the assumption that this material is produced and made available, what are the pertinent issues to be considered? These are new issues to consider in the framework of government regulation, assurance of effectiveness, consumer acceptance, agronomic potential, and most importantly what are the allergenic consequences of the use of low allergen crops? It is this host of questions and the resulting answers that will either facilitate or limit the use of allergen-reduced crops as a means of mitigating allergy. Soybean has the potential to be an important test for this potential on whether allergen-reduced or -eliminated crops can be used to mitigate the effects and growth of food allergies.

Developing allergen-reduced crops as single gene alterations for soybean and other crops is now feasible. For soybean over 50% of the allergencity is P34 and if this were to be stacked with Kunitz Trypsin Inhibitor, Lectin, and perhaps Gly m bd 28k nulls this would eliminate 80 to over 90 % of the allergenicity of soybean.With conventional germplasm accessions available for all of these allergens, stacking these together is simply a matter of crossing and selecting appropriate segregants. Although simple in principle, crossing plants with tiny flowers and selecting for individuals with several independent traits that must be self-crossed and selected until a stable homogenous population is reached, all with a plant with a 140 day life cycle, is a long and laborious process. Several years of selective breeding will go into this goal now undertaken by collaborating laboratories including that of this author.

With the near-term potential to have low allergen soybeans available for testing and eventual use many new questions will emerge. The first important question is how effective would such a soybean be in mitigating existing soybean allergy and what would the consequences of such a soybean be in eliciting the development of new soybean allergy. The correlation of IgE binding capacity and allergic response may or may not mitigate the response, so eliminating major IgE binding proteins in soybean still needs to be tested physiologically. Ideally the model would be that without the major IgE binding proteins soybeans lacking these proteins should provoke little or perhaps no response and therefore be tolerated by otherwise sensitive people. However would this tolerance be permanent or would people consuming products made from low allergen soybeans still acquire sensitivity to yet other soybean constituents? Both animal and human testing will be necessary to test the paradigm that food can be made less or not allergenic by modification of allergen content. Animal testing in particular will be critical because by using animals with no prior exposure to soy, a direct comparison can be made with wild type soybeans containing the full inventory of allergens with soybeans that are null for a majority of the proteins that elicit. Will such soybeans fail to produce the development of an allergenic response, which is the outcome hoped for? Follow-on tests with human subjects will then be necessary to confirm the animal results. If successful, there will be applications of these soybeans in products such as soybean-based infant formula. Infant formula allergy is a major concern with 8% of all infants developing allergy to bovine milk and/or soybean-based formula. About half of the infants allergic to bovine milk formula will also develop soybean-based formula allergy.

Once shown effective at mitigating the allergenic response and the development of allergy, there are many further significant regulatory and legal questions that will need to be addressed. The question of labeling such products

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under current US law under the The Food Allergen Labeling and Consumer Protection Act requires soybean to be plainly labeled as such and there is no provision on how a lower allergen or allergen-null soybean might be labeled to conform to the act. This question will need to be addressed by the appropriate authorities and the decision process to assure effectiveness of the products in mitigating allergies and issuing approvals will likely take a number of years. As the data are accumulated and testing occurs, a wide range of discussions with government, industry, and consumer groups will be necessary to move low allergen soybeans from the laboratory to product. If we look toward the future and imagine a product such as low allergen infant formula sitting on a market shelf there will be many questions of regulation, approval, and effectiveness that will be necessary so that the consumer reaching for that bottle of formula is assured that the translation of the laboratory concept for a low allergy food into a consumer product meets the expectations necessary to help alleviate the growth in allergic disorders.

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Chapter 29

Improvement of Immunoassays for the Detection of Food Allergens

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The development of immunoassays for the detection of food allergens is a challenging task because the target is usually a complex mixture of allergenic and non-allergenic proteins of diverse structural and chemical properties. Moreover, the detection of these proteins might be affected differently by food processing. Consequently, it is difficult to predict which proteins are actually being detected by the immunoassay. Indeed, the protein profile in the sample extract might not be fully comparable to that of the calibrators, which brings up the question of accuracy in a quantitative assay. A better characterization of extracts from allergenic foods is needed in order to standardize, optimize, and validate the detection methodology. This chapter will highlight some of the proteomic tools to optimize the detection of food allergens.

In the US, the Food Allergen Labeling and Consumer Protection Act (FALCPA) requires all products containing major allergenic foods (milk, egg, soy bean, wheat, peanut, tree nuts, crustaceans, and fish and their products) to be indicated in the ingredient list. Analytical methods are needed to detect the inadvertent presence of allergens in processed foods. There are commercial immunoassays available for only some of the eight major allergenic foods, none of which have been formally validated. Only some peanut kits have undergone a performance evaluation under the AOAC Research Institute program (*1*). Validation is needed to ensure the specificity, accuracy, and precision of the kits and to guarantee reproducible results. All these elements provide a solid basis

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for making labeling decisions. Commercial peanut kits are quantitative tests but they differ in their ability to provide the same result for the same sample. In our research we have evaluated, at the molecular level, some of the factors responsible for this variation and the possible options to help reduce inherent uncertainty of these kits. The understanding of these factors is necessary to improve and standardized detection methods and they should be considered in the planning of the validation process.

Understanding the Target

The most important factor in the development of any detection assay is the understanding of the nature of the target. Every compound has specific properties determined by its physical and chemical characteristics, which govern how the different steps in the analysis process are designed and optimized. Proteins are a heterogeneous group of large molecules that have very different roles in nature. The activity of proteins is determined by their 3-dimensional structure. Food allergens are proteins, but not all the proteins are allergenic. It still remains uncertain why only some proteins are allergens and why sensitive individuals are allergic to some but not all of them.

Food Allergens as Targets

Food allergens as targets are not like any other biological markers. Biological targets like the prostate specific antigen (PSA), used as a marker for prostate cancer, are always detected as intact molecules. However, an additional and very important factor has to be considered in the development of detection assays for food allergens, and that is food processing. Food is a defined mixture of ingredients that can undergo different chemical (acidification, fermentation), physical (heating, extrusion, microwaving, high pressure, irradiation), or enzymatic treatments. During the production of foods the different food components are modified or they interact with each other resulting in a final product characterized by its specific texture, flavor, color, and moisture, among other properties. The processing of food may cause the irreversible modification of some individual ingredients, including proteins (*2*). Like any other component food allergens are also affected by food processing (*3, 4*) and this may have an important impact in the analytical process. Food allergens can form chemical cross-links to other food components, such as sugars in the so-called Maillard reaction (*5, 6*), or to other proteins resulting in insoluble aggregates (*7, 8*). They can be hydrolyzed as a consequence of enzymatic or acid treatment. Allergens can be also denatured by heat-producing treatments (*9*). As a result of food processing, allergen solubility may be reduced, impacting extraction efficiency

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during sample preparation. Moreover the 3-dimensional structure of an allergen may be modified affecting its recognition by the antibodies in an immunoassay.

Food allergens are not equally affected by the same processing treatment because some allergens are more resistant than others to specific treatments. It is very difficult, if not impossible, to predict how much a protein is affected by food processing because it depends on the treatment conditions and the food composition. These vary from product to product, from brand to brand, and from lot to lot. In other words, the protein being analyzed in the final food can be, in fact, very different from the intact form present in the raw material and detection methods need to be developed accordingly. Without an understanding of this concept, it is impossible to comprehend the complexity of the detection of food allergens.

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Making Sense of Immunoassays for Food Allergens with Proteomic Techniques

Antibody-based assays (immunoassays) have been used as the methods of choice for the detection of food allergens not only because of their specificity and sensitivity but also because they target the offending allergen. It is for this same reason that PCR techniques are not so widely accepted. PCR targets DNA that encodes the allergenic protein, but it is not the actual trigger of the allergic response. The detection of allergen-encoding DNA does not necessarily correlate with the presence of allergens in the sample. This is particularly true in cases where the food has been fortified with purified or semi-purified protein.

Immunoassays can be found in different formats, microtiter plate (ELISA), lateral flow device, and biosensor and they can use monoclonal, polyclonal antibodies, or a combination of both. Immunoassay formats and applications have been extensively reviewed (*10*). Immunoassays can target one or several proteins. Most of the commercial kits developed for the detection of food allergens use polyclonal antibodies. However, they differ in specificity and the number of proteins they target. Some of them target one or two food allergens, while others target all the protein content of the sample, including allergen and non-allergens. What these three formats, ELISA, lateral flow device, and biosensor, have in common is that they generate a positive signal when the target or targets are being detected. They do not provide information about which individual target is detected. The ability to monitor which analyte is being detected is crucial to understanding how the immunoassay performs when different samples are being analyzed and the suitability of an individual assay for the detection of allergens in processed foods.

To characterize and fully understand the detection of allergens, from sample preparation to the data generated by ELISA, we have used techniques commonly used in proteomic studies such as sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), two-dimensional electrophoresis, and immunoblots.

Sample Preparation

Sample preparation is as critical as the analytical technique. If both steps are not optimized the accuracy of the analysis will be jeopardized. The purpose of sample preparation is to extract the analyte from the sample with a minimum of interfering compounds. Moreover, the sample extraction solution used has to be compatible with the immunoassay and it has to preserve the structure of the analyte so that it is detected by the antibodies.

As mentioned above, food processing impacts the analysis of food allergens. Processing may modify the allergen and affect its solubility. Sample preparation deals with the solubility factor and the sample extraction buffer plays a very important role in solubilizing the allergens prior to detection. Solubility can be reduced due to denaturation and the formation of insoluble protein aggregates. Unfortunately, some of these changes are irreversible resulting in a reduced protein recovery. For example, roasting of peanuts reduces the solubility of peanut allergens (Figure 1) in general terms. However, because every allergen has different physical-chemical properties it is essential to talk about differential solubility. The major peanut allergen Ara h 1, which is one of the most abundant proteins in peanuts, tends to aggregate with roasting (*8*) and its solubility is drastically reduced when comparing extracts from light to dark roasted peanuts. Another major peanut allergen, Ara h 2, has a more stable structure (*11*) and, even though is not as abundant as Ara h 1, it becomes one of the major components in dark roasted peanut extracts (Figure 1).

Extraction buffers differ in their ability to extract food allergens (*12*). Extraction efficiency can be qualitative and quantitative. Regarding the quantitative aspect of extraction solutions, some solutions extract the same allergens but in different concentrations. Alkali solutions have been shown to be more efficient than high salt buffers at neutral pH in the extraction of peanut proteins (*12*). Regarding the qualitative aspect, not all the extraction solutions can extract all the allergens. For example, phosphate buffer, a common buffer used in immunoassays, fails to extract the major peanut allergen Ara h 3, a globulin. This protein is efficiently extracted by the same buffer containing salt (Figure 2 - 2D gels loaded with phosphate buffer and high salt buffer peanut extracts). Thus, it is crucial to select the right extraction solution for the target or targets of interest since the accuracy of the assay depends on the extraction efficiency of the solution.

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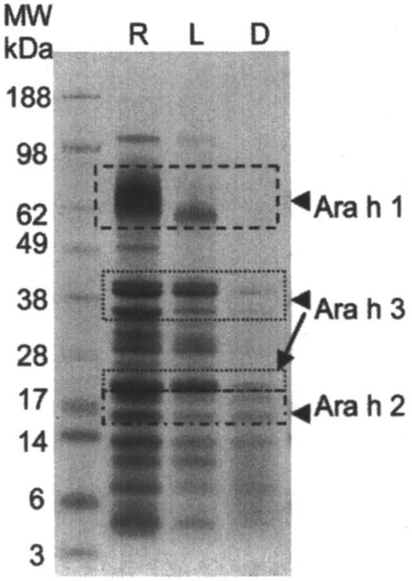

Figure 1. Comparison of protein content in raw peanut (R), light (L), and dark (D) peanut flour by SDS-PAGE.

Immunoassay

The selection of commercial immunoassays for food allergens should be based on performance. Performance should be understood at the molecular level, where the real detection occurs, not simply by the evaluation of final results. However, these elements of commercial assays are unknown even to scientists involved in their evaluation because of the proprietary nature of commercially available reagents and the fact that many of these reagents are only partially characterized. As a result, the criteria for selection of commercial immunoassays by the final user is limited to price and time of analysis. It is very difficult to compare commercial ELISAs not only because they use different extraction procedures, but also because the antibodies may have different specificities (*12*). Another variable is the lack of standardized reference materials used in these kits. However, the suitability of several potential reference materials have been evaluated, like NIST peanut butter (*13*), NIST egg powder (*14*), and gliadin (*15*).

To evaluate immunoassays it is necessary to understand the complexity of three independent elements that need to be integrated in a way that allows for optimal assay performance. These three elements, in addition to the sample extraction buffer (described above), are the following:

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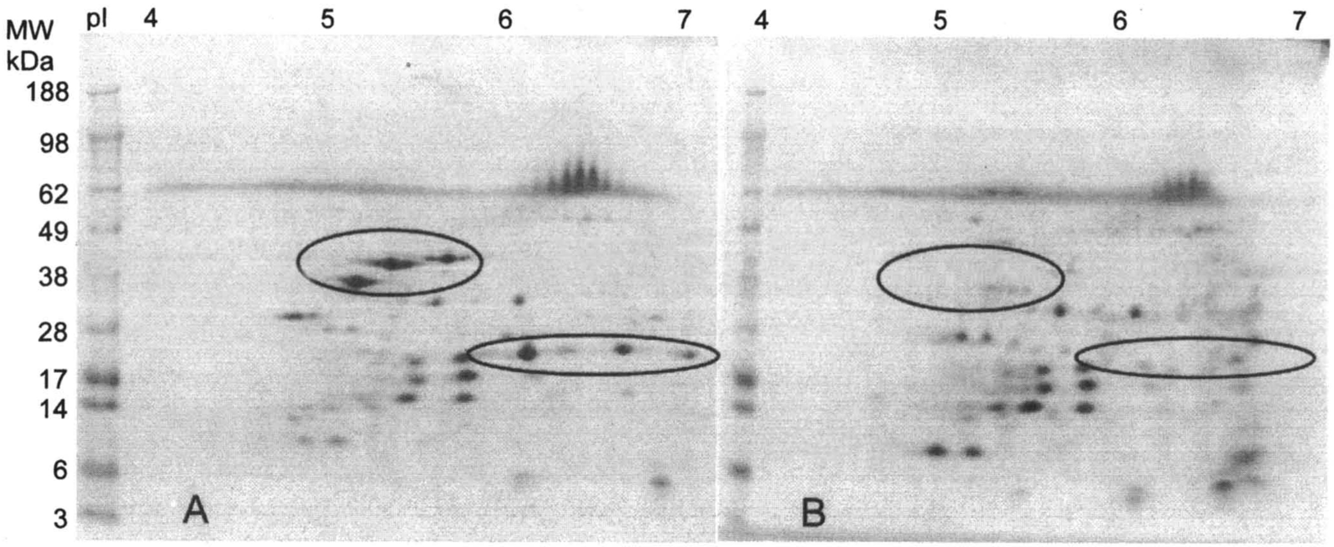

Figure 2. Evaluation, by 2-D electrophoresis, of the ability of (A) high salt buffer and (B) phosphate buffers to extract the peanut allergen Ara h 3 from raw peanuts

- The target, which can be a single allergen, multiple allergens, or proteins.
- The antibodies used for detection need to be produced once the target or targets have been selected. They can be monoclonal or polyclonal.
- Reference material used to create the standard curve which will be compared to that of the sample for quantitative purposes.

In the evaluation of immunoassay performance it is basically impossible to study the three elements separately because the outcome of the assay is the result of their interaction. In this chapter the standard curve will be used as the focus for explaining the ELISA and its sources of variability and uncertainty.

The design and characterization of the standard curve and its correlation to the targeted components in a sample are key elements in the accuracy and reliability of a quantitative immunoassay. It is a pre-condition that the standard curve contains the target of interest. However, the target may be physiochemically altered depending on how it was processed. An advantage of working with antibodies is that the antibodies can be developed so that they bind allergens in both the raw material and processed foods. Because proteins are in different proportion in food with respect to each other and with respect to the total food, and because they have different susceptibilities to different food processes, it not easy to find a single reference material that represents all the types of foods. Immunoassays only use one standard curve and it has to be selected after a careful evaluation of different available reference materials. Ideally, the result of this evaluation should be that the standard curve is the same regardless of the degree of processing. This can be accomplished by using the right antibody for the right target. In other words, if an immunoassay includes three standard curves made of three different reference materials, it would be ideal that the resulting curves overlap (Figure 3A). In a situation where the same assay uses different standard curves and they do not overlap, it would be very difficult to decide which curve is representative of the sample (Figure 3B). This has a negative impact in the quantification process.

To better understand the role of antibody specificity in the suitability of different reference materials as candidates for standard curves, antibodies of different specificity were used to compare different standard curves prepared with two different peanut materials – light and dark roast peanut flour.

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Detection Based on Multiple Targets

- Case 1. Standard curves are evaluated with polyclonal antibody (pAb), specific for 3 allergens (Figure 4B), one of reduced solubility after roasting (Ara h 1) and two that are more soluble (Ara h 2 and Ara h 6). The drop in signal of dark peanut flour is basically due to the reduced presence of Ara h

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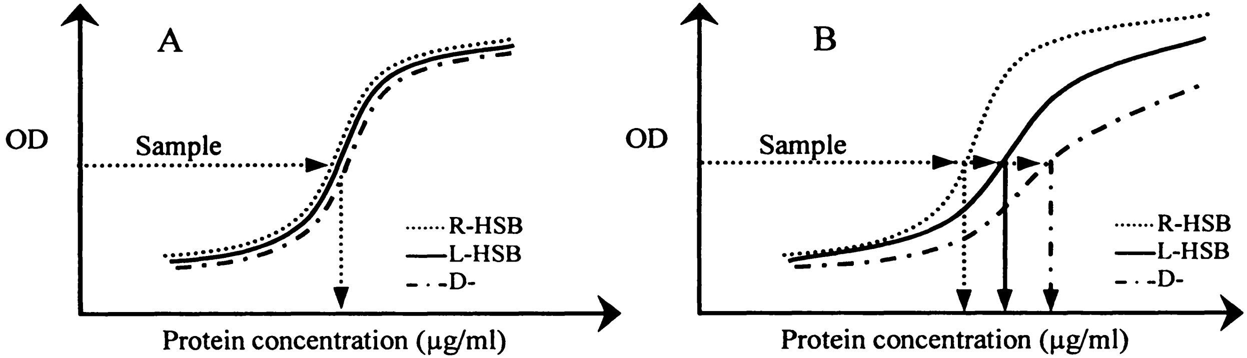

Figure 3. Consequences of not using the proper antibody specificity in immunoassay using three different reference materials raw peanut (R-HSB), light (L-HSB), and dark (D-HSB) roast peanut flour: (A) standard curves overlap providing the same result for the same sample or (B) standard curves do not overlap providing different results for the same sample.

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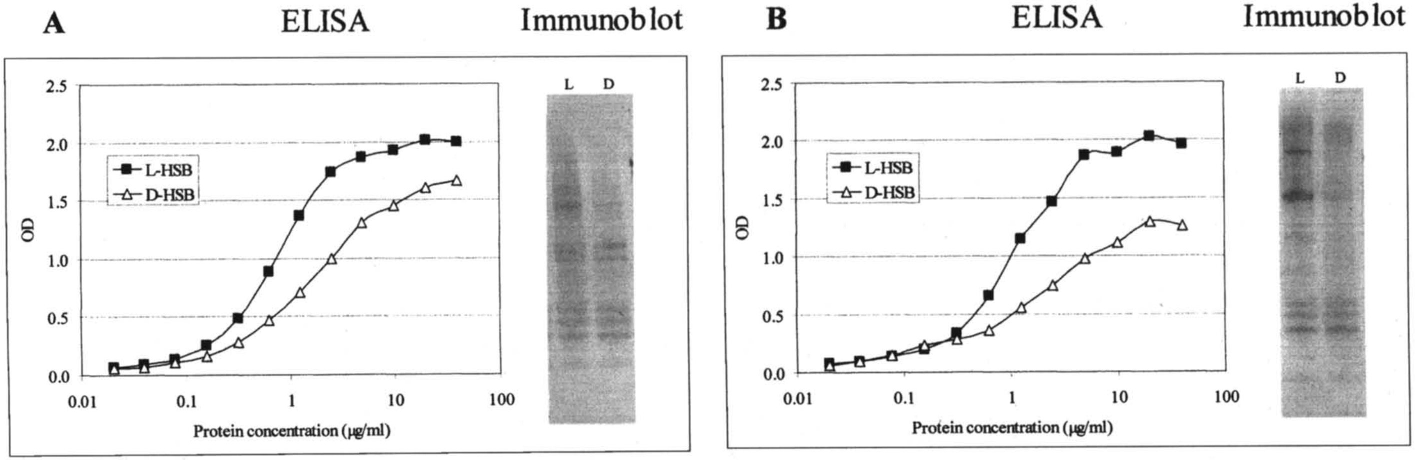

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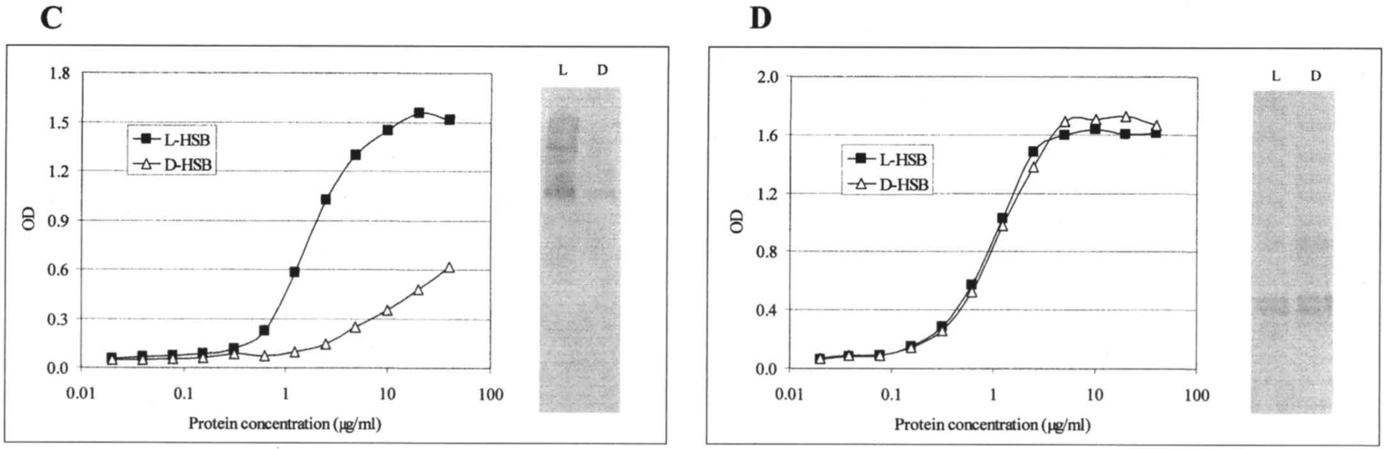

Figure 4. Effect of antibody specificity on the standard curve when targeting (A) multiple proteins,, (B) peanut allergens Ara h 1, Ara h 2 and Ara h 6, (C) Ara h 1, and (4) Ara h 2, on the signal generated from both light (L) and dark (D) roast peanut extracts used as reference material in ELISA.

1 in the extract. In other words, the assay is missing one of the antibody's targets (Ara h 1) and the detection of dark roast peanut flour is based on the detection of Ara h 2 and Ara h 6. Situations like this may result in quantitative inaccuracies.

- Case 2. This case is more complex because it is based on the detection of the majority of peanut proteins (Figure 4A). Even though there are missing targets in the dark roast peanut flour extract, the difference between curves is not as drastic as in case 1 because there are still additional target proteins in the more hightly processed sample.

In these two examples, the standard curves made with light and dark roast peanut flour extracts do not overlap and this may result in quantitative errors. The main reason for this lack of overlap is that the use of antibodies with specificity for multiple targets results in a lack of consistency in the number of targets detected in different processed samples. The impact of a situation like this is not apparent because when samples are analyzed only a single standard curve is used and the resulting signal generated for the sample is forced to fit in this standard curve.

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Detection of a Single Target

- Case 3. By targeting a single protein the analyte is known at all times and it is for this reason that the detection is better controlled. The selection of an appropriate target must be based on its stability and solubility after processing. For these reasons, the peanut allergen Ara h 2 appears to be a good candidate (Figure 4D).
- Case 4. As shown in Figure 4C, Ara h 1 is not a good target because of its reduced solubility in dark roast peanut extracts. Thus, Ara h 1 is not an optimal target for detection.

The detection of food allergens based on a mixture of targets with different degrees of susceptibilities to treatments should be carefully evaluated as an option. In general terms, the most labile allergens should be eliminated as targets because even though these proteins may be detected in the extract used to generate the standard curve, they would remain absent or at very low levels in extracts from food that underwent harsh processing conditions. This type of target is a source of inadvertent inaccuracy and uncertainty of detection methods when used in a multi-target detection system, and, more dramatically, when they are used as single targets. Another consequence resulting from the use of insoluble targets is the high probability of a false negative assay result from a hightly processed sample.

Data Processing for Quantification Purposes

The final concentration of the food allergen depends on several factors that need to remain constant for quantification purposes. These factors are the following:

- Standard curve – it is constant for the set of samples analyzed along with the standards.
- Dilution factor – It refers to the predetermined volume of sample extraction buffer used to extract the sample. For example, for 10 ml of sample extraction solution per gram of sample, a factor of 10 is used to calculate the concentration of the allergen per gram or mL of sample. The extraction procedure is constant and it is predetermined by every kit manufacturer. In other words the dilution factor should be constant for each kit.
- Conversion factor – In addition to the factors of assay variability mentioned above, which include antibody specificity and reference material, commercial kits are also difficult to compare because they use different reporting units. Even though immunoassays detect food proteins, many of the commercial kits report the results as the concentration of the allergenic food in the sample instead of the concentration of the targeted food allergens in the sample. For example, one kit detects peanut proteins and it reports the results as ppm peanut, or another kit detects the egg allergen ovomucoid but the results are reported as ppm egg white proteins or ppm egg. The conversion factor is a value based on the total protein content or allergen in the food group, and it is usually calculated by Kjieldahl or Dumas tests. For example, in the case of raw peanut, 25% (or ¼) of the peanut corresponds to the protein content, therefore a factor of 4 is used to report the results as ppm peanut. However, in immunoassays we analyze soluble protein not total protein. Because protein solubility is inversely proportional to the degree of processing of the sample, the conversion factor should increase with the degree of processing of the sample. For this reason, the conversion factor cannot be constant. Because we cannot determine the degree of processing of the blind sample, we cannot determine the value for the conversion factor that should be applied to that particular sample. The use of a conversion factor introduces more uncertainty in the final result.

Conclusions

New regulations for the labeling of food allergens are leading to a proliferation of immunoassays. There are neither criteria for their development nor common reference materials. Moreover, there is no agreement regarding the

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reporting units. These factors are responsible for the result variability between kits. In order to comply with national or international regulations and to enforce these regulations both the food industry and regulatory agencies need to have validated detection methods. However, before the validation process can proceed, the source of variability in the kits should be evaluated and addressed in order for error to be reduced or eliminated. How is this possible? The evaluation of the final result of a kit should be complemented by the information provided by looking at every step of the analytical process with proteomics technology. Tools such as SDS-PAGE or two-dimensional electrophoresis and immunoblots are unique instruments to complement immunoassay detection by monitoring targets and antibody specificities. They can be used in early stages of the development of immunoassays as well as in the preliminary evaluation of kits before validation takes place. The more that is known about all the reagents and elements of the assay the more controlled the detection process will be:

- It may not be possible to accurately quantify food allergenic proteins in processed samples due to changes in protein solubility. However, error can be minimized by selecting the appropriate sample extraction buffer.
- The design and characterization of the standard curve as well as correlation of standard proteins to the targeted proteins in a sample extract are key elements in the accuracy and reliability of a quantitative immunoassay. These elements are a function of specificity of the antibody used in the assay. An ideal detection system would be the one that provides the same result regardless of the chosen reference material. This can be achieved by selecting the right antibodies for the right targets. Detection cannot rely on targets that are unstable or have poor solubility after processing, but on targets where solubility is not jeopardized.
- The introduction of conversion factors to modify the reporting units may introduce inaccuracies to the final results and therefore uncertainty to the overall detection. Because kits may use different reporting units it is nearly impossible to compare results from different commercial test kits.

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Chapter 30

Detection of Gluten by Commercial Test Kits: Effects of Food Matrices and Extraction Procedures

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To investigate the effects of food matrices and extraction procedures for different test kits used for determination of gluten, six commercially available test kits were evaluated: R-Biopharm RIDASCREEN® Gliadin, RIDASCREEN® FAST Gliadin, RIDAQUICK Gliadin, Neogen Veratox® for Gliadin, Tepnel BioKits Gluten Assay kit, and Morinaga Wheat Protein ELISA kit. The food matrices used were gluten-free guar gum and high-fiber bread mix, also spiked with different concentrations of gluten standard reference material (SRM 8418) obtained from National Institute of Standards and Technologies (NIST). In addition, 30 gluten-free samples were surveyed for gluten content. A homogeneity study showed that spiked gluten reference materials were evenly distributed in food matrices. Because sample extraction mixtures used by the kits are different, extraction efficiencies were carefully evaluated, especially for Neogen and R-Biopharm kits, which provide the same two options for sample extraction, aqueous alcohol and cocktail buffer. Results obtained with the six assay systems and extraction procedures differed considerably. Extraction with cocktail gave almost two times higher concentration of gluten than that from ethanol extraction using RIDASCREEN kits. Further investigation of the kits resulted in divergent findings. Depending on test methods or extraction procedures used, false positives and negatives were observed.

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This variation creates uncertainty to end users. There is an urgent need for harmonization of the use of reference materials in control standards, and validation of extraction procedures in order to define the analytical results.

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Wheat is the staple cereal in many countries and its uses in manufactured foods are ever growing due to the technological qualities of gluten proteins as well as the numerous applications of different wheat fractions. Cereal grains, providing approximately half of the world's supply of human dietary protein, are also used as ingredients in processing aids, such as thickening agents, fillers, and binders, and they can be found in baked goods, processed meat products, confectionery items, and drinks, among many other processed foods. Although wheat is widely consumed, the ingestion and inhalation of wheat proteins may trigger different disorders in sensitive individuals. Wheat allergy is an IgE-mediated reaction to wheat proteins, and can manifest as a classical food allergy syndrome affecting skin, gut, respiratory tract, or cardiovascular system (*1*). In addition, ingestion of wheat proteins may also cause celiac disease, a T-cell mediated intestinal inflammation (*2*). The different wheat protein fractions involved in the pathogenesis of these distinct disorders are well characterized (*3*). While albumin is found to be the primary fraction of importance in baker's asthma, gluten protein fractions are toxic to individuals suffering from both wheat allergy and celiac disease.

Dietary glutens, the major storage proteins in cereal grains of wheat, rye, and barley, can be potentially toxic to individuals with celiac disease (*4*). Gluten is a highly heterogeneous group of proteins, and consists of both aqueous alcohol-soluble prolamins and alcohol-insoluble glutelins. Prolamins can be isolated from wheat (gliadins), rye (secalin), barley (hordein), and oats (avenin). Like food allergies, the prevalence of the disease is higher than previously thought, which has become a public health issue (*5*). It is believed that the prevalence of celiac disease ranges from 1 in 250 (*6*) to 1 in 133 (*7*) in the United States. The disease is genetically determined, with 10% of the first degree relatives affected and 75% of monozygotic twins being concordant (*8*). Gluten proteins are strongly associated with DQ2 and DQ8 human leucocytes antigen (HLA) class II haplotypes (*9, 10*) and can cause the loss of the villous structure of the small intestine therefore compromising the absorption of nutrients. The classic symptoms of celiac disease consist of diarrhea and weight loss in adults and failure to thrive in children because of nutritional deficiencies. Strict avoidance of toxic concentrations of gluten in the diet is the only effective treatment for celiac disease individuals.

In the United States, the Food Allergen Labeling and Consumer Protection Act of 2004 (FALCPA) requires the US Food and Drug Administration (FDA) to define the term "gluten-free". Therefore, a validated quantitative analysis of gluten in foods is desperately needed by food industries and regulatory agencies to ensure the accuracy of the labels, which will ultimately protect celiac disease individuals.

Immunoassays, such as enzyme-linked immunosorbent assay (ELISA) and lateral flow device (LFD) for gluten, are specific, sensitive, and rapid detection methods. Currently, in the United States, about 10 immunoassay kits for gluten/wheat are commercially available. Among these kits, there are about 4 different gluten detection antibodies: R5 monoclonal antibody (*11*) used in kits manufactured by R-Biopharm and Ingenasa (*12*), Skerritt monoclonal antibody (*13*) used by Tepnel, Hallmark, and Diffchamb (*14*), two different monoclonal antibodies used by Neogen Corporation in the Veratox gliadin kit, and a polyclonal antibody used in the Morinaga wheat protein kit (*15*). While the European reference standard for gliadin, IRMM-480 from the Institute of Reference Materials and Measurements (IRMM), is not yet commercially available, test kit manufacturers have been using different wheat or gliadin materials as their control standards. In addition, it is very confusing to the end users that components of extraction procedures, such as extraction buffers, temperatures, and incubation times, vary widely among detection kits (Table I). Whether these variables affect the repeatability and reproducibility of the results obtained from different detection kits needs to be further investigated. To address these issues, we evaluated six commercial immunoassay gluten kits to study the effect of different specificity of antibodies, control standards, food matrices, and extraction procedures in determination of gluten levels in food.

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Methods

Immunochemical Test Kits

The gluten test kits used in this study were RIDASCREEN Gliadin, RIDASCREEN Fast Gliadin, and RIDAQUICK Gliadin from R-Biopharm AG (Darmstadt, Germany), Veratox for Gliadin from Neogen Corporation (Lansing, MI), BioKits Gluten Assay Kit from Tepnel BioSystems (Deeside, UK), and Morinaga Wheat Protein from Morinaga Institute of Biological Science Inc (Japan) and distributed in the US by Crystal Chem (Dowers Grove, IL). The assay procedures were carried out according to the instructions from manufacturers. The parameters of each kit are summarized in Table I.

Sample Preparation

To examine the effect of food matrix in the recovery of gluten, a 10 mg portion of gluten obtained from National Institute of Science and Technologies

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Table I. Commercial Gluten Test Kits Used

Company	Neogen Corp.	R-Biopharm AG			Tepnel Biosystem	Morinaga Inc.
Product	Veratox	RIDASCREEN	RIDASCREEN Fast	RidaQuick[a]	Gluten Assay	Wheat Protein
Antibody	2 mAb	R5 mAb	R5 mAb	R5 mAb	Skerritt mAb	Wheat pAb
Target Antigen	gliadin	α γ ω-gliadin	α γ ω-gliadin	α γ ω-gliadin	ω-gliadin	Wheat proteins
LOD (ppm)	n/a	3	10	5[b]	1	0.3[c]
LOQ (ppm)	10	5	10	n/a	10	3.12[c]
Extraction Procedure	40% ethanol, or cocktail + 55% alcohol	60% ethanol, or cocktail + 80% alcohol	60% ethanol, or cocktail + 80% alcohol	40% ethanol	40% ethanol	Denaturing and reducing buffer, overnight

[a] Lateral flow device.

[b] Cut-off value for a qualitative assay.

[c] Concentrations are ppm of wheat protein.

LOQ = limit of quantification; LOD = limit of detection; n/a = data not available.

mAb = monoclonal antibody; pAb = polyclonal antibody.

(NIST), gluten SRM 8418, was spiked into 200 g of gluten-free high-fiber bread mix, guar gum, and 200 mL of matrix-free 60% ethanol, and thoroughly mixed to make 50 ppm of gluten in the samples. Both high-fiber bread mix and matrix-free 60% ethanol were further diluted in the same matrix to give final concentrations of 25, 10 and 5 ppm of gluten. Homogeneity of the spiked high-fiber bread mix, at 50 and 25 ppm levels, was confirmed by analyzing four portions of each sample using the Veratox for Gliadin kit. All analytical samples were prepared using the sample extraction buffer provided by the kits. In the case of Neogen and R-Biopharm kits, the two suggested mixtures were used for extraction - aqueous ethanol and cocktail. The samples were analyzed according to the procedures of the test kit used.

We also surveyed 30 gluten-free samples obtained from the gluten-free market as well as four gluten-containing products as positive controls. Samples were analyzed using the aqueous ethanol extraction procedure from the Neogen kit.

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Statistical Analysis

The levels of gluten in samples were calculated either from the software provided by manufacturers or by linear and non-linear regression in Prism® 3.0 (San Diego, CA). R square < 0.98 required the analysis to be repeated.

The homogeneity of spiked samples was statistically analyzed by one-way ANOVA using MINITAB program (State College, PA). The material was considered homogeneous when $P > 0.05$.

Results and Discussion

Gluten is a highly heterogeneous group of proteins, and consists of prolamins and glutelins that can be isolated from a number of grains such as wheat, corn, rice, rye, barley, and oats (*16*). But only gluten from wheat, rye and barley has been associated with reactions of celiac disease (*8*).

Due to the need for having reliable methods to confirm compliance with the requirements for gluten-free labeling, we evaluated six commercial kits (four brands). Table I summarizes the performance characteristics of the commercial test kits on the US market. This information is public or it has been provided by the manufacturers. Not only do the kits differ in antibody specificity but also in the sample extraction procedures. Every kit provides its own sample extraction mixture. Its composition varies from different percentages of aqueous alcohol to buffers containing detergent and reducing agents, or a combination of both in a 2-step extraction. Because the two gluten fractions, prolamins and glutelins,

Table II. Homogeneity Study of Spiked High-fiber Bread Mix Using Veratox for Gliadin

Gluten Spiked in High-fiber Mix	*Mean ± Std.Dev. (ppm)*	*No. of Tests*	P *value*[a]
25 ppm	23.5 ± 1.8	4	0.27
50 ppm	46.0 ± 4.5	4	0.23

[a] One-way ANOVA used to analyze homogeneity. Samples were homogenous when *P* values were greater than 0.05.

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have different solubility properties in these two types of buffer, our evaluation of the kits focused on extraction efficiency in different food matrices.

Spiked Sample Homogeneity

The first step in the study was to prepare the samples containing different levels of gluten from high-fiber bread mix spiked with gluten NIST standard SRM 8418 at a final concentration 50 ppm; this level was further diluted with the same mix to provide samples containing 25, 10, and 5 ppm gluten. To ensure that the mixing procedure was properly carried out and to avoid variability due to a non-homogeneous sample, four portions of each 50 and 25 ppm gluten samples were analyzed with Veratox Gliadin kit. Results shown in Table II indicate that the samples were homogeneous at the two gluten levels and they could be used in our study.

Determination of Matrix Effect in Blank Samples

In this study, two gluten-free samples, guar gum and high-fiber mix bread, in addition to 60% ethanol, were used to investigate the effect of food matrix on the determination of gluten. Whether the model food matrices were extracted by aqueous ethanol or cocktail buffer, no gluten was detected using RIDASCREEN, Neogen or Tepnel kits (Table III). However, Morinaga wheat protein kit showed presumptive positives above the LOQ of 3.13 ppm in both, the gluten-free guar gum (3.3 ppm wheat protein) and the high-fiber bread mix (3.8 ppm wheat protein), but not in matrix-free 60% ethanol. This result must be interpreted with caution. There are two possible explanations for these values. First, the limit of quantitation (LOQ) of the Morinaga kit is the lowest of the six kits evaluated in this study. It is possible that the LOQ has been calculated by manufacturer from wheat proteins in buffer or food samples different from the ones used in this study, therefore in this situation we could certainly say that

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Table III. Effects of Food Matrices, Extraction Procedures and Detection Systems in Determination of Gluten or Wheat Protein Levels in Spiked Samples

Spiked Gluten (ppm)	*Matrix*	*RIDASCREEN Fast Gliadin (LOQ 10 ppm Gluten)*		*RIDASCREEN Gliadin (LOQ 5 ppm Gluten)*		*Rida Quick (Cut-off 5 ppm)*	*Veratox (LOQ 10 ppm Gluten)*		*Tepnel (LOQ 10 ppm Gluten)*	*Morinaga (LOQ 3.12 ppm Wheat Protein)*
		Ethanol Gluten (ppm)	*Cocktail Gluten (ppm)*	*Ethanol Gluten (ppm)*	*Cocktail Gluten (ppm)*	*Ethanol Gluten (ppm)*	*Ethanol Gluten (ppm)*	*Cocktail Gluten (ppm)*	*Ethanol Gluten (ppm)*	*Morinaga Buffer Wheat Protein (ppm)*
0	Guar gum	ND	ND	ND	ND	-	ND	ND	ND	3.3
50	Guar gum	ND	26.3	16.4	30.4	+	41.8	50.2	55	54.7
0	Bread mix	ND	ND	ND	ND	-	ND	ND	ND	3.8
5	Bread mix	ND	ND	ND	ND	+	ND	ND	ND	9.5
10	Bread mix	ND	ND	ND	ND	+	13.2	12.8	ND	17.8
25	Bread mix	ND	ND	ND	ND	+	26.0	22.2	21.5	34.2
50	Bread mix	16.2	32.6	15.5	39.3	+	46.0	50.8	42.5	58.7
0	60% EtOH	ND	ND	ND	ND	-	ND	ND	ND	ND
5	60% EtOH	ND	ND	ND	ND	+	ND	ND	ND	5.7
10	60% EtOH	ND	ND	ND	7.2	+	11.0	12.4	11.2	14.7
25	60% EtOH	ND	12.2	ND	13.1	+	23.6	23.2	21.0	25.9
50	60% EtOH	20.8	30.4	17.4	36.3	+	51.0	45.0	39.4	52.6

LOQ=limit of quantification; ND=not detected.

these positives are due to matrix effects. The second possibility is that the samples are really contaminated with very low levels of gluten, which are not detected by the other kits because they have higher LOQs. The Morinaga kit uses a sample extraction procedure different to those of the other kits. Even though the Morinaga extraction buffer contains a reducing agent and a detergent, like cocktail buffer, they are more concentrated. Moreover, samples are extracted for longer periods of time (overnight up to 12 h) in contrast with the other kits with extraction times up to 100 min (in the case of cocktail). It is unknown if extended extraction times or higher concentrations of the reducing agent and the detergent lead to the extraction of additional proteins or any other component from the matrix responsible for the positive results. A sensitive alternative method like polymerase chain reaction (PCR) or mass spectrometry would be very helpful in deciding whether this positive is due to contamination or matrix effect. Controversial situations, like this one, are examples where the availability of confirmatory methods plays a critical role in the analysis of gluten.

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Effects of Extraction Procedures in Gluten Recovery

For the evaluation of gluten recovery from food matrices we selected NIST gluten SRM 8418 as reference material because it is available to all, and there is no lot-to-lot variation. Gluten contents of the different spiked samples are summarized in Table III.

For the Veratox gliadin kits, all levels of gluten spiked in all three matrices used were detected with accuracy around 100%, even at the LOQ level of 10 ppm in buffer and bread mix. Moreover, no positives were found at 5 ppm, which is below the LOQ of the kit. No differences between ethanol and cocktail were observed. Similarly, Tepnel also detected almost all levels of gluten in all matrices. However, it was not able to detect gluten at the LOQ level of 10 ppm of gluten in bread mix. This is an expected result since we cannot expect to have 100% gluten recovery. Even if the recovery is within the accepted range of 80-100% it is very possible that gluten remains undetected in this sample.

Three different immunoassays from R-Biopharm were evaluated: two quantitative ELISAs developed in a 96-well microtiter plate format and a third one which is a qualitative lateral flow device, with a cut-off value of 5 ppm gluten that distinguishes between a positive and a negative sample. They use the same monoclonal antibody R5 so similar results are expected. Surprisingly, both RIDASCREEN ELISAs only detected gluten in the 50 ppm samples with about 32% recovery in ethanol extracts versus 70% for the cocktail. Only cocktail extracts showed positives in 60% ethanol spiked with 25 ppm gluten, but recoveries barely reached 50%. Given these results, there are two aspects of recovery that need to be discussed. First, the overall low recovery of gluten from the samples by the two RIDASCREEN ELISAs may not be related to an extraction efficiency problem. We need to be aware that the reference material used by the manufacturer is different from the NIST gluten reference material that we used to spike the food samples in terms of gluten content and gluten proteins. The manufacturer calibrated their control standards against the European reference standard IRMM-480 which is definitely different from NIST SRM 8418. The second aspect of the recovery that needs to be addressed is based on the differences found between the two different sample extraction buffers. In the case of the RIDASCREEN ELISAs, cocktail extracts provided about 2-fold gluten levels compared to ethanol. This observation has been previously reported (*17, 18*). Garcia et al. (*17*) found that cocktail yielded gluten levels between 1.1- and 3.0-fold higher than aqueous ethanol in the analysis of unheated products and wheat starch. Different results provided by the same kit with the same sample only create confusion, uncertainty, and questions about the real gluten content in the sample. And more importantly, results like this can create a make-or-break situation in a gluten-free claim regardless of the action levels provided by different regulatory agencies.

Both Neogen and R-Biopharm kits provides cocktail or aqueous ethanol as options for sample extraction. Aqueous ethanol has been traditionally used for the extraction of prolamins. However, after the food has been heated, some of the prolamin proteins tend to aggregate, becoming insoluble in this solvent mixture. Cocktail extraction solution contains guanidine hydrochloride, a disaggregating agent, which along with a reducing agent helps solubilize these proteins. At the time of the study, no recommendations were offered as to when alcohol or cocktail should be used. Currently, both Neogen and R-Biopharm recommend cocktail for the analysis of gluten from heated foods such as baked goods. For unprocessed samples, the gluten content determined by the two kits and the two extracts should be similar. Furthermore, even though the resulting gluten content provided by the two kits is different, the ratio of gluten content in cocktail and aqueous ethanol extracts determined by one kit should be the same as that provided by the second kit. However, this is not the case because the cocktail/ethanol ratio for R-Biopharm is 2 while it is 1 for Neogen. The reason for a different ratio is probably due to differences in antibody specificity. R-Biopharm uses a single antibody R5 and it has been well characterized and documented. We know that Neogen uses two different monoclonal antibodies (personal communication) but the information about their characterization has not been made public. This type of information is critical to understanding the dynamics of the detection as well as the interpretation of results.

Surprisingly, the RidaQuick LFD, which also uses mAb R5, seems to be more sensitive than the ELISA counterpart. It was able to detect gluten in all of the matrices at all levels, including at the 5 ppm cut-off value, but not in the blanks. In our hands, this LFD provides most reliable results for all the samples that we have analyzed, provided that it fits the purpose of the testing.

Regarding the Morinaga kit, extraction efficiencies reported for all levels in all samples are around or over 100% in most of the cases that could be due to a

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matrix effect as discussed above. Values are very similar to those provided by Neogen. However, similar values do not mean same results because reporting units makes the difference. The Neogen kit reports the results as ppm gluten while the Morinaga kit reports the results as ppm wheat proteins, so even though the numbers are the same they cannot be compared. It is not exactly known what type of conversion factor Morinaga uses to convert ppm of gluten to ppm wheat proteins, if wheat proteins are defined by the manufacturer as the totality of wheat proteins.

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Detection of Gluten in Commercial Gluten-free Foods

We surveyed 30 commercial gluten-free foods, grouped in more than 8 food categories, as well as four gluten-containing products (Table IV). All the analyses were carried out with the Neogen Veratox kit. Among the gluten-free samples, only buckwheat flour had detectable gluten (46.6 ppm). However, four different lots of this gluten-free buckwheat flour were purchased from the same company at different times and no detectable gluten was observed. To better characterize the source of contamination, the sample was analyzed by PCR by an independent lab. The flour was positive for wheat, and negative for rye and barley; oat was not tested by the PCR analysis.

The contaminated buckwheat flour sample was subject to further analysis by the six kits (Table V). If the results observed in the previous study with spiked samples can be generalized, we would expect lower values with R-Biopharm ELISAs than the rest of the kits. However, results did not follow that pattern. Results for cocktail RIDASCREEN tests are not only higher than the rest, but also are negative for the aqueous ethanol extract, like Tepnel. Neogen also had the same result for both extracts with similar values for Morinaga, analogous to the results from the recovery study. These types of results that do not follow the same pattern are quite disturbing, and there are two possible explanations. One possibility is that the wheat residue is not homogeneously distributed in the bag of buckwheat flour. The second possibility relates to the antibody specificity. We do not know if the antibodies used in these kits have different affinities for gliadins from different wheat cultivars and how different are those to the ones used in control standards. This can certainly cause deviation from the general pattern of results, i.e., that kit 1 always provides twice the result values from those of kit 2. These divergent results are puzzling and make accurate and reliable detection very difficult. This variability will have a bearing on the ability to perform meaningful risk assessments.

Conclusion

In this study, six antibody-based gluten detection kits from four companies were evaluated in different matrices and were further used for the determination

Table IV. Determination of Gluten Content in Foods with Neogen Veratox

Sample Type	*Food Category*	*No. of Samples*	*Gluten (ppm)*
Gluten-free	Mix	5	ND
	Flour	6	ND
	Buckwheat flour	5	ND/46.6[a]
	Sauce	4	ND
	Noodle	2	ND
	Bread	2	ND
	Cracker	2	ND
	Rolls	2	ND
	Bagel	2	ND
Gluten-containing samples	Flour	1	816
	Waffle	1	1817
	Cereal	1	1809
	Bread	1	1979

[a] One out of 5 buckwheat flour packages (same brand, different lots) was found positive. ND=not detected.

of gluten content in gluten-free food samples. Extraction efficiency of extraction mixtures from the kits was carried out in food matrices spiked with NIST gluten reference standard SRM 8418. Results differed from assay to assay. It is difficult to perform a full assessment of the kit performance just with the results provided by the kits. In light of the divergent results, it seems clear that there is a need for standardization of reference standards and sample extraction procedures for the detection of gluten to minimize the potential sources of variability. In fact, almost every aspect of the kits should be carefully evaluated, including sample extraction buffers, type of antibody (monoclonal versus polyclonal), a single antibody versus a combination of them, antibody specificity, reference material, and reporting units. With the information we currently have we do not know which is the best approach or which elements from the different kits are introducing uncertainties and to what extent this uncertainty contributes to the final result. Some of the aspects of these kits can only be clarified by the manufacturers. In order to really compare the kits we need to understand how they were designed, developed, and optimized. Moreover, more research is needed to understand the target analyte(s). In the case of gluten the kits are targeting multiple proteins, which may vary with the type of cultivar. Furthermore, we need a better understanding and characterization of the modifications to the target analyte(s) produced during the production of food. Standardized reference materials suitable for the detection of gluten in food

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Table V. Gluten Concentrations found in a Gluten-free Buckwheat Flour

	RIDASCREEN Fast Gliadin		*RIDASCREEN Gliadin*		*Rida Quick*	*Veratox*		*Tepnel*	*Morinaga*
Extraction mixture	Ethanol	Cocktail	Ethanol	Cocktail	Ethanol	Ethanol	Cocktail	Ethanol	Buffer
Target analyte	Gluten	Gluten	Gluten	Gluten	Gluten	Gluten	Gluten	Gluten	Wheat protein
Concentration (ppm)	ND	37.5	ND	> 80	Positive	46.6	49.8	ND	59.0

ND=not detected.

ingredients and processed foods are desperately needed. For all of these reasons we cannot conclude which kit is the most accurate and the best performer.

There is an imperative need for accurate gluten results, which can only be obtained if reliable and validated detection methods are available. The issue has to be addressed with urgency since regulatory agencies are defining the "gluten-free" term and they are proposing rules for its use. "Gluten-free" is a critical action level or threshold that will ensure the health of millions of individuals suffering from celiac disease as well as to maximize their food choices. Clearly, monitoring and risk assessment programs can be jeopardized if the detection issue is not addressed.

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Chapter 31

Easy Analysis of Allergen Residues in Food: A New Lateral Flow Test and an ELISA

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Food allergen testing is becoming more important, not only because of legislation in the USA and Europe, but also because of in ever increasing customer interest. With this, the need for easy, fast and reliable test systems arises. Herein, we introduce new test systems for detection of allergens, developed by R-Biopharm, which address these needs: a lateral flow test for detection of hazelnut protein and for peanut proteins usable in a wide variety of foods and also a competitive ELISA for gliadin. The gliadin test is specially designed for detection of fragments of gluten in highly processed foodstuffs as starch, syrups, or beer.

Allergen residues in food have become a more important topic over the last few years. In addition to the demand by allergic individuals for proper labeling to avoid adverse affects due to lack of knowledge of the presence of allergens, different legislation around the world regulate allergen labeling of processed food. Within the USA the FALCPA (*Food Allergen Labeling and Consumer Protection Act*) defines the so called "Big 8" – milk, egg, fish, crustacean shellfish, tree nuts, wheat, peanuts, and soybeans – as the major cause of allergic reactions to food. Their presence in foodstuff has to be listed on the food labels. In the European Union, Directive 2003/89/EC amends an earlier directive on food labeling with a list of 12 allergens, which have to be listed explicitly and independently of their concentration on the ingredients list. These allergens are the same eight as in the USA plus celery, mustard, sesame, and sulfur dioxide (SO_2) only when exceeding a concentration of 10 mg/kg or L).

The need to label allergens also generates the need to know about their presence or absence in food products by the food industry as well as by control

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authorities. This demand requires the development of analytical methods and kits for determination of these allergens. The task is made especially difficult by two facts. First, there are no reference methods or materials to compare new test kits or methods and second, as no thresholds or limits exist (except for SO_2 in the European regulation), these methods have to be as sensitive as possible and should work in all kind of food matrices.

On one hand, the nature of the analytes, allergens, which are mostly proteins, make antibody based methods a prime candidate. On the other hand, nowhere in the regulation is the presence or absence of the allergenic compounds of a foodstuff explicitly mentioned, a close to impossible task anyway as a great number of proteins within a certain food can act as an allergen and this might be different from individual to individual. So any other method which can determine the presence or absence of the listed food could be used also, e.g., molecular biological methods like PCR (polymerase chain reaction).

R-Biopharm, with its long tradition in development of antibody based analytical methods, such as ELISA (enzyme-linked immunosorbent assays) and immunochromatographical tests (lateral flow devices, LFDs), developed a range of test kits for determination of allergens in foodstuffs. The two recent additions to R-Biopharm's range of allergen test kits are a lateral flow test for hazelnut and a competitive ELISA for gliadin.

Lateral Flow Devices for Determination of Hazelnut and Peanut

A research project sponsored by the European Union within the 5th Framework (project ALLERGENTEST, QLRT-2000-001151) brought together partners from 6 different European nations to develop rapid tests for the determination of peanut and hazelnut in different foods. First, new specific monoclonal antibodies to hazelnut and to peanut proteins were produced which later on were used in the development of lateral flow devices (LFD). The marketing and distribution of these products is performed by R-Biopharm.

The basis of a lateral flow device (LFD) is an immunochromatographic assay principle with an antigen-antibody reaction using specific capture and detection antibodies (see Figure 1). Results are read visually by observing the development of colored bands. In the hazelnut test, the reagents migrate through the membrane of the reaction strip. Each reaction strip contains a membrane, which is coated with specific anti-hazelnut antibodies. In case of a hazelnut content of the sample, the hazelnut antigen will bind to beads, coated with anti-hazelnut antibodies. The bead-hazelnut complex will be carried to the part of the membrane which is coated with anti-hazelnut antibodies. The bead-hazelnut complex will be bound by the anti-hazelnut antibodies and a blue band becomes visible in the field of reaction of the test strip (zone 3). No false negative results (no high

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dose hook effect) will occur, if high concentrations of hazelnut are employed (e.g. 1 g of hazelnut or 100% hazelnut). Unbound colored beads pass the field of reaction and will be absorbed in the absorption pad at the end of the membrane (zone 4). Additionally, beads (dark gray circles) with a control substance will form another blue band as a control band for a correctly working test (basic antigen/antibody reaction).

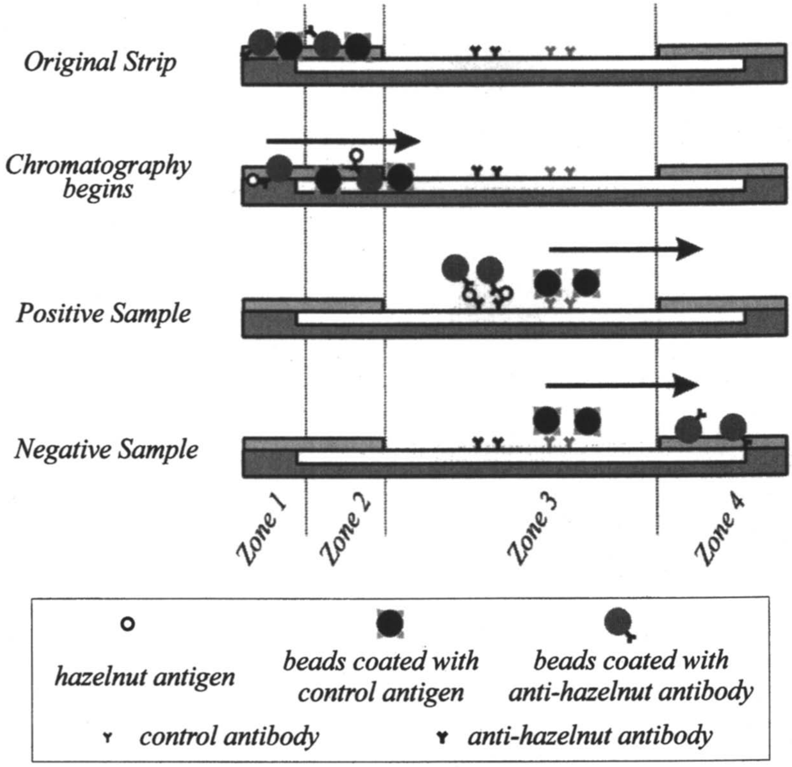
Original Strip
Chromatography begins
Positive Sample
Negative Sample
Zone 1
Zone 2
Zone 3
Zone 4
hazelnut antigen
beads coated with control antigen
beads coated with anti-hazelnut antibody
control antibody
anti-hazelnut antibody

Figure 1. Principle of a lateral flow test for allergen: migration of hazelnut through a RIDA®QUICK Hazelnut

Different kinds of matrices (chocolate, cookies, ice cream, sausages, salad dressing, instant soup, corn flakes, and yoghurt) were spiked with different levels of hazelnut (in the range of 2.5 - 35 ppm hazelnut). The samples were checked for homogeneity after filling into bottles. Twenty five blind coded samples were sent out, and extracted according to the leaflet (1 g sample plus 1 g skim milk powder are added to 20 mL pre-heated extraction buffer and incubated 10 min at

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60 °C (140 °F)). After centrifugation, aliquots are transferred to the LFDs and the results are read visually or with the use of a reader after 10 min. All tests were done in duplicate. The appearance of a single control line shows the test was working fine but no protein, hazelnut or peanut, depending on the test, was found in concentrations above the cut off. Cut offs were set to about 5 mg/kg of respective nuts. If a second line is visible after the 10 min incubation time, a positive sample, containing more then 5 mg/kg nuts was found. No lines at all indicate a failed test and the analysis has to be repeated.

The tests were validated in two different studies. One, a collaborative study by 8 European laboratories, compared the results of homemade spiked samples. Twenty three samples, blind coded, were sent out and measured in duplicate by the participating laboratories. The second validation was done in cooperation with consumer protection agencies from 11 European countries that collected a total of 440 samples in their respective markets. The range of matrices covered cookies, dark chocolate, milk chocolate, cereals, instant soups, and sausages. Both studies showed excellent correlations between laboratories and also between results from the LFD and prototype ELISA (developed during the project). Negative, unspiked samples tested negative in both systems. A comparison between the RIDASCREEN®FAST Hazelnut ELISA and the RIDA®QUICK Hazelnut LFD using some spiked samples is shown in Table I.

Table I. Comparison of ELISA and RIDA®QUICK Hazelnut on different samples

Determination of spiked samples (results in mg hazelnut/kg product)

Sample	*Spike*	*ELISA* Results*	*LFD results after 10 min* *Test line*	*Control line*
Homemade chocolate + raw HN**	5	5.8	+	+
Homemade chocolate + roasted HN	5	5.4	+	+
Ice cream + roasted HN + skim milk powder	5	5.2	+	+
Butter cookies + roasted HN + skim milk powder	5	4.5	+	+
Salad dressing + roasted HN + skim milk powder	5	4.6	+	+
Cheese + roasted HN + skim milk powder	5	4.5	+	+
Cereals + roasted HN + skim milk powder	5	5	+	+
Dark chocolate + roasted HN	5	5.3	+	+
Cookies + roasted HN + skim milk powder	5	5.1	+	+

* RIDASCREEN®FAST Hazelnut (R6802), R-Biopharm, Germany

** HN: hazelnut

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Competitive ELISA for Determination of Gliadin in Highly Processed Foods

Gliadin, the prolamine in wheat, is an important protein in wheat. Prolamines are part of the so-called gluten fraction of cereals. Gluten is on one hand important for baking quality of wheat, on the other hand a major health threat for people suffering from celiac disease, an autoimmune disorder of the small bowel. Therefore, the Codex Alimentarius sets a limit of 200 ppm as maximum concentration of gluten in foodstuffs to be safe for consumption by celiac patients. Naturally gluten-free foods derived from cereals (e.g. buckwheat, sorghum) which are not toxic to celiacs are only allowed to contain 20 ppm gluten.

ELISA based analytical systems are used for determination of gluten in food. Actually, only the prolamine, as the soluble part of gluten, is analyzed and the resulting gluten concentration is calculated. Unfortunately, some foods contain highly processed cereal proteins, e.g., beer, starches, or syrup, making it rather difficult to get good analytical results. These hydrolyzed proteins do not allow the use of classical sandwich ELISA methods for determination of gliadins. This is an additional problem to the problems with extraction of the prolamines from heat treated food ,which only can be done by using a special extraction cocktail.

Different publications (*1-3*) showed that a 33 amino acid peptide from gliadin with the sequence LQLQPFPQPQLPYPQPQLPYPQPQLPYPQPQPF is resistant to gastric and pancreatic hydrolysis and acts as a strong stimulator to intestinal T cells. It is speculated that it is the toxic peptide for celiac patients. Sub-sequences of this peptide were used to check for their reactivity with the R5 antibody (*4*, *5*). This antibody is internationally recognized as the most fitting for determination of the gliadin content in foodstuffs. Finally, a small peptide – QQPFP – which is recognized by this antibody was selected for the development of the competitive gliadin ELISA.

In the test kits, microplates are coated with a fixed amount of gliadin. The samples and an enzyme-conjugated anti-gliadin antibody (R5 antibody) are pipetted into the microwells. Hydrolyzed peptide fractions of natural gliadin (peptides of different length, depending on the degree of hydrolysis, down to the smallest fragments of 5 amino acids that the antibody can recognize) compete with the gliadin on the microwells of the plate for the added conjugated antibodies. The assay is calibrated to the QQPFP peptide, found as one of the repeatable motifs of the 33-mer epitope, which the R5 antibody recognizes very strongly (*4*).

The more natural gliadin or peptide is present, the more of the added conjugate is bound by it and the less of it can bind to the microplate, resulting in a weak color development. Negative samples, containing no gliadin or peptides can not bind any of the conjugate so all the antibody binds to the microwell resulting in an intensive color development.

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Table II. Comparison of Gliadin Results of Sandwich ELISA and competitive ELISA (results in ppm (mg/kg))

Sample	*Sandwich ELISA**		*Competitive Assay***
Extracted	*Ethanol*	*Cocktail solution*	*Ethanol*
Wheat starch 1	10.5	15.2	44.3
Wheat starch 2	26.2	23.5	32.7
Syrup 1	< LOD	< LOD	< LOD
Syrup 2	< LOD	< LOD	8.6
Beer 1	< LOD	< LOD	9.6
Beer 2	9.4	-	21.7
Wheat beer 1	1,500	960	3,150
Wheat beer 2	716	-	977

* RIDASCREEN® Gliadin (Ref. Nr. R7001), R-Biopharm, Germany

** Prototype of RIDASCREEN® Gliadin competitive, R-Biopharm, Germany; results are expressed as ppm gliadin units in this case

Studies using hydrolyzed starches, syrups, and beer showed that in many cases notably higher “gliadin concentrations”, compared to analysis using the classical sandwich ELISA, could be found (see Table II). In these tests, the results are expressed in mg gliadin per kg sample; future commercial tests will give results as ppm peptide, which will give much higher values.

However, it is still not completely understood of what relevance these results are for celiac patients and more work is necessary to correlate the findings of a competitive gliadin ELISA with gluten contents and more importantly with pathogenic impacts of foodstuffs analyzed.

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Chapter 32

Characterization of Gluten Proteins in Grain Flours by Liquid Chromatography–Tandem Mass Spectrometry Using a Quadrupole Time-of-Flight Mass Spectrometer

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Grain flours from a local bulk food store were analysed using liquid chromatography Quadrupole-Time of Flight tandem mass spectrometry for the proteins in the ethanol soluble gluten fraction after extraction and enzymatic digestion. Glutens from wheat, rye, barley, and oat were readily identified using a database search of the MS/MS data. Prolamins were also detected in "gluten-free" flours such as rice and corn. Extracts of multi-grain mixtures were analysed and the major glutens present were easily identified.

Glutens are a class of proteins found in cereal grains. Glutens present in wheat, barley, and rye are believed to trigger celiac disease (CD) which is a life long autoimmune intestinal disorder found in genetically susceptible individuals. When individuals with CD ingest gluten, the villi in the small intestine are damaged and become ineffective in absorbing basic nutrients such as proteins, carbohydrates, fats, vitamins, and minerals (*1, 2*). If CD is left untreated, the damage can be chronic and life threatening. Wheat gluten is also a major contributor to wheat allergies. Allergic reactions may be caused by ingestion of wheat-containing foods or by inhalation of flour containing wheat (Baker's asthma) (*3*).

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Glutens are found in the alcohol soluble fraction of the flour and are made up of two groups of proteins, the prolamins and glutelins. The prolamins, which include the gliadins from wheat, hordeins from barley, avenins from oat and secalins from rye, are monomeric polypeptide chains with molecular weights ranging from 30 to 50 kDa and rich in proline and glutamine. Glutelins include low molecular weight (LMW) and high molecular weight (HMW) varieties with molecular weights ranging from 15 kDa to 150 kDa, but some of the HMWs can go as high as 3,000 kDa. They contain a high number of disulphide bonds that are responsible for the elasticity of the bread dough. Glutelins are also high in proline and glutamine.

Currently, Enzyme-Linked Immunosorbent Assay (ELISA) kits are used to detect glutens in food (*4*). These methods are constantly being modified and new kits are being developed to improve the effectiveness of the detection of glutens, especially wheat gluten (*5*). However, many of these kits also nonspecifically detect the presence of barley, rye and oat prolamins. In addition, some of these commercial kits are based on monoclonal antibodies that react with a specific type of gliadin – the ω-gliadins. Studies have shown that in different varieties of wheat, there are significant differences in the relative percentages of other gliadin species such as the α-gliadins and the γ-gliadins, which vary in their affinities for the antibodies used in these kits and may produce ambiguous results (*6*).

In recent years, mass spectrometry has gained popularity in the analysis of proteins in food (*7, 8*). Matrix-Assisted Laser Desorption Ionization (MALDI) mass spectrometry has been explored for use in the determination of protein composition in different wheat varieties (*9*) and in differentiating between gliadins of different wheat varieties (*10*). MALDI was also investigated in the analysis of gluten in food (*11*).

This paper describes the use of a nano liquid chromatography (LC) system coupled to a hybrid Quadrupole-Time of Flight (Q-TOF) mass spectrometer to analyse a tryptic digest of the alcohol soluble fraction of flour extracts. The MS/MS data obtained from this system is searched against the NCBI protein database (a public resource database of protein sequences from the National Center for Biotechnology Information) and proteins are identified and characterized based on their peptide fragments.

Experimental

Reagents

Ethanol (EtOH) used in the solvent mixture for extraction of gluten from flour was obtained from Commercial Alcohol Inc. (Brampton, Ontario). Baker

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Analyzed HPLC Reagent grade water from Mallinckrodt Baker Inc. (Phillipsburg, NJ) was used in the preparation of the extraction solvent mixture for the flour as well as solvent for LC. OmniSolv HPLC grade acetonitrile (ACN) was obtained from EMD Chemicals Inc. (Gibbstown, NJ) for LC. Ammonium bicarbonate, dithiothreitol (DTT), iodoacetamide (IA), and formic acid (FA) used in the digestion protocol were obtained from Sigma Chemical Co. (St. Louis, MO). Sequencing grade trypsin was obtained from Promega, (Madison, WI) for the proteolytic digestion of the protein.

Sample Preparation and Extraction

One gram of flour was extracted with 10 mL of 60% EtOH/water (v/v) in a 50 mL centrifuge tube. The mixture was placed on a rotary shaker at 380 rpm for 1 h at room temperature and then centrifuged for 30 min at 11,000 rpm at 22 °C using an Eppendorf Centrifuge model 5804R (Brinkmann Instruments, Inc., Westbury, NY). The clear supernatant layer was transferred to a sample tube and the residue was discarded. A 1 mL aliquot of the sample was further serially diluted 10 times and 100 times with the extraction solvent.

Proteolytic Digestion with Trypsin

Proteolytic digestions were carried out using a multiprobe robotic liquid delivery system, MassPREP STATION (Waters, Milford, MA) for the fully automated reduction, alkylation, and digestion of proteins. All reagents were freshly prepared prior to digestion. Thirty µL aliquots of the sample were placed in a 96 well v-bottomed microtitre plate. Then a 15 µL volume of DTT (10 mM DTT in 50 mM ammonium bicarbonate) was added to each sample to reduce the disulphide bonds and the plate was incubated at 37 °C for 30 min. The mixture was then allowed to cool to room temperature (10 min). A 15 µL volume of 55 mM IA in 50 mM ammonium bicarbonate was added and the samples were incubated at room temperature for 20 min. Trypsin (300 ng) in 50 mM ammonium bicarbonate was added to each well and incubated at 37 °C for 3 h. Finally, 15 µL of 1% FA was added to the digested mixture. The resultant peptide mixtures were transferred to micro-centrifuge tubes and centrifuged for 20 min at 14,000 rcf at room temperature using an Eppendorf Mini Spin Plus centrifuge (Brinkmann Instruments Inc., Westbury NY). The clear supernatant layer was transferred to a 250 µL autosampler vial ready for LC-MS/MS analysis.

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LC-MS/MS Analysis of the Digested Mixture

The LC-MS/MS system used was a Waters (Waters, Milford, MA) CapLC system interfaced to a Quadrupole-Time of Flight (QTOF2) mass spectrometer (Waters) through a 10-port stream selector. The LC system consisted of a trap column (OPTI-PAK 0.35 mm x 5 mm packed with Waters Symmetry 300 C18 5 µm for pre-column sample cleanup and an analytical nanocolumn (75 µm i.d.) for peptide separation. The analytical column used was 100 mm x 75 µm packed with 5 µm PepMap C_{18} 100 Å (LC Packing/Dionex, Sunnyvale, CA). A capillary (0.1 mm x 150 mm) silica monolithic column Onyx C_{18} (Phenomenex, Torrance, CA) was also tested. A binary solvent gradient (A+B) with an effective column flow of 200 nL/min was used for the analysis of the tryptic digests. Solvent A was 3% ACN/H_2O with 0.2% FA and solvent B was ACN with 0.2% FA. The mobile phase was initially 5% solvent B, increased at 0.8%/min to 35% B, followed by 0.5%/min to 45% B, then back to 5% B at 60 min. The system was allowed to equilibrate for 10 min at a flow rate of 5 µL/min with 5% B before the next injection. The total run time was 70 min.

The mass spectrometric data were acquired using a MassLynx 4.0 data system (Waters). The mass spectrometer was operated in nanoelectrospray positive ion detection mode with a spray voltage of 3.5 kV at a resolution of 10000 (FWHH). Accurate mass was achieved through the correction using a reference (Glu1-Fibrinopeptide B (Sigma) infused at 25-50 fmol/min) from mass spectra acquired alternately between the reference and analyte sprays. The acquisition mode was data directed analysis (DDA), acquiring MS survey data from *m/z* 350 to 1400 with the criteria to switch to MS/MS mode based on intensity threshold and charge state (+2, +3 and +4). A detailed description of the system can be found in a previous study using LC-MS/MS to identify and detect trace milk allergens in food (*8*).

Protein Identification Using Database Search

The LC-MS/MS data produced a list of precursor ions with their corresponding MS/MS fragments. This list was submitted to a search engine and the MS/MS spectra were matched to protein databases. The bioinformatics software package used in this work was from Matrix Science (Matrix Science Inc., Boston, MA) and included Mascot Server (an in-house licensed database search engine), Mascot Distiller (a peak detecting software which processed the raw data into de-isotoped peak lists) and Mascot Daemon (a client application which automated the submission of data files to Mascot server allowing batch mode, real time monitoring, and score-dependent follow up activities such as conditional repeat searches). The peak list created from the raw LC-MS/MS data

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with Mascot Distiller (after accurate mass correction from the reference scan data) was subjected to a Mascot MS/MS Ions Search which looked for the best peptide sequence match to each MS/MS spectrum. Peptide matches were then grouped into protein matches. Protein identification is based on the Mascot score which is a probability based scoring system based on the Mowse algorithm with the formula: Mowse score = -10 x log(p), where p represents the probability that the observed match is a random event. Mascot calculates the probability of the match between the experimental data and the expected mass values of a peptide or protein sequence being a random event. The correct match, which is not a random event, would then have a very low probability.

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Results and Discussion

Flour samples were purchased from a local retail bulk food store. Fourteen different grain flours plus two flour mixtures were characterized using this method. These are commonly available flours for use in making bread, noodles, and pasta. The wheat derived flour included whole wheat flour (soft and hard); Durum wheat flour from Durum which is a high protein hard wheat used in making pasta; Durum semolina (from endosperm of Durum) used to make macaroni and spaghetti; gluten flour (wheat flour treated to remove most of its starch leaving mainly gluten) used as an additive to dough made of low-gluten flours such as rye flour. Spelt is a sub-species of common wheat. Spelt flour contains gluten but seems to be tolerated by many people with gluten allergies. Barley, rye, and oat flours were chosen in this study because they are known to contain gluten; some of these cause reactions in individuals with CD. Some of these glutens also give cross reactions with ELISA kits designed to detect wheat gluten. Corn, rice (white and brown), and buckwheat (light and dark) were declared as gluten free by the food retailer. Finally, a six grain bread mix and a seven grain flour mix were used to test the ability of the method to detect and differentiate glutens from different species present in a mix. The six grain mix contained wheat bran, barley flour, corn meal, cracked buckwheat, rolled oats, and rye meal. The seven grain flour indicated wheat, rye, triticale, barley, corn, millet, and soy on the ingredient list.

The major proteins detected in the flours tested are indicated in Table I. The proteins from grain are generally classified according to their solubility. The classification of wheat protein is shown in Table II (*12*). Albumin is soluble in water. Both albumin and globulin are soluble in dilute salt solution. Gliadins, found in wheat gluten, are generally soluble in 60% ethanol-water solution. The separation based on solubility is not absolute, therefore proteins from other fractions overlapped into the gluten fraction as demonstrated by the presence of the water soluble amylases and amylase inhibitors (the albumin and globulin proteins of wheat flour) shown in Table I. In the wheat-related flours, glutens from wheat were identified which included the various homologs of gliadins -

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Table I. Major Proteins Detected Using LC-MS/MS in the 60% Ethanol Fraction of Grain Flours

Flour	*Major Proteins Detected in the 60% EtOH Fraction*
Soft whole wheat	Gamma-gliadins, LMW glutenin, HMW glutenin, D hordein, alpha-amylase inhibitors
Hard whole wheat	Gamma-gliadins, LMW glutenin, HMW glutenin, alpha-amylase inhibitors
Durum flour	Gamma-gliadins, LMW glutenin, HMW glutenin, alpha-amylase inhibitors
Semolina Durum	Gamma-gliadins, LMW glutenin, HMW glutenin, alpha- amylase inhibitors
Gluten flour	HMW and LMW glutenin, gamma-gliadin, D hordein
Spelt (organic)	Gamma-gliadins, LMW glutenin, HMW glutenin, Alpha-amylase inhibitors
Barley (stone ground)	Barley dimeric alpha-amylase inhibtors, B3 hordein, B1 hordein, D hordein, gamma-3 hordein
Rye (dark)	Secalin precursor, alpha-amylase inhibtors (wheat), D hordein, HMW glutenin
Oat	Avenin, avenin precursor, LMW glutenin subunit
Rice (white)	Prolamin *Oryza sativa*, 13 kDa prolamin, allergen RA14B precursor rice
Rice (brown)	Prolamin *Oryza sativa*, 13 kDa prolamin, allergen RA14B precursor rice, etc.
Corn	19 kDa alpha zein D2, beta-zein precursor, 15kDa beta-zein
Buckwheat (dark)	BWI-1 protease inhibitor, major allergenic storage protein [*Fagopyrum esculentum*]
Buckwheat (light)	BWI-1 protease inhibitor, major allergenic storage protein [*Fagopyrum esculentum*]
Six grains bread mix	Gamma-gliadins, LMW glutenin, HMW glutenin, B3 hordein, alpha-amylase inhibitors
Seven grain	Gamma-gliadin, secalin precursor, B3 and D hordeins, putative vacuolar protein rice

Table II. Protein Fractionation of Wheat Proteins Based on Solubility

Protein Class	*Soluble in*	*Comments*
Albumin	Water	pH is important
Globulin	Dilute salt solution	
Gliadin	Water, 60% EtOH	Low solubility in water
LMW glutenin polymers	Water, SDS (sodium dodecyl sulfate) solution, 70% EtOH	Small oligomers only, low solubility in water
HMW glutenin polymers	1.5% SDS	Applies to HMW glutenin <500 kDa
Glutenin macro polymers	Insoluble in the solvents listed above	Soluble after reduction or sonication

the prolamins and the glutelins (LMW and HMW glutenins). In addition, hordeins (gluten from barley) were also detected in the soft whole wheat flour. A typical search result from Mascot for the soft whole wheat protein digest is shown in Figure 1(a-c) with the top 10 significant hits (1-a), followed by the score distribution chart of the proteins identified (1-b) and the detailed information about each protein that was identified, including the protein accession number, protein description, protein mass, protein score, and peptides that were identified (1-c). The error of the masses (difference between the theoretical peptide mass and the observed peptide mass in daltons) was shown in the column labeled "Delta". This shows the mass accuracy of the acquired MS/MS data and reflects on the confidence level on the search result. The search results of the protein summary were exported into a spreadsheet. A portion of the spreadsheet from the soft whole wheat extract is shown in Table III. The wheat glutens found were gamma-gliadin, gamma-gliadin precursors, alpha/beta-gliadins, the LMW glutenin subunits and some HMW glutenin subunits. Other proteins such as protease inhibitors, lipid transfer proteins and proteins from the water and salt soluble fractions were also detected. Some of these proteins were easily digested by trypsin as seen from the high percentage of protein coverage from the amylases. Since trypsin only cleaves proteins at the carboxyl side of the amino acids lysine (K) and arginine (R) except when either is next to a proline (P), it may not be the optimum enzyme to use for the digestion of the prolamins which contain an average of 20% proline. In addition, neither K nor R is very abundant in the prolamin sequence. However, trypsin is commonly used in proteomics because of its well defined specificity and it is useful for obtaining a whole protein profile of the sample.

Protein summaries for barley, rye, and oat flour are shown in Tables IV, V, and VI. The prolamins found in barley flour include B hordein, B3 hordein, D hordein, gamma-3 hordein, gamma-2 hordein and B hordein precursors. Other

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Table III. Protein Summary from MASCOT MS/MS Ion Search Result of the Soft Whole Wheat Flour Extract

Protein Accession no.	*Protein Description*	*Protein Score*	*Protein Mass*	*Protein % Coverage*
gi|54778503	0.19 Dimeric alpha-amylase inhibitor [*Triticum aestivum*]	584	13827	83.1
gi|123968	Alpha-amylase inhibitor 0.53	422	13690	58.9
gi|123966	Alpha-amylase inhibitor 0.28 (CIII) (WMAI-1)	359	13888	55.3
gi|17425170	LMW glutenin subunit group 3 type II [*Triticum aestivum*]	330	42464	20.2
gi|57470935	Alpha-amylase inhibitor precursor [*Aegilops speltoides*]	317	13956	42.7
gi|45477539	LMW glutenin pGM107 [*Triticum aestivum*]	287	38876	17.6
gi|17425182	LMW glutenin subunit group 3 type II [*Triticum aestivum*]	283	34262	19.1
gi|21920	CM2 protein [*Triticum turgidum* subsp. *durum]*	259	16020	53.1
gi|7230478	Gamma-gliadin [*Triticum aestivum*]	254	35632	21.4
gi|607198	15 kDa grain softness protein [*Triticum aestivum*]	193	17670	27.1
gi|225103	Trypsin/amylase inhibitor pUP38	182	12417	29.6
gi|121103	Gamma-gliadin B precursor	163	33402	21.6
gi|21743	HMW glutenin subunit 1Ax1 [*Triticum aestivum*]	140	89993	4.5
gi|21711	CM 17 protein precursor [*Triticum aestivum*]	131	16549	24.5
gi|18918	Unnamed protein product [*Hordeum vulgare*]	130	59895	4.5
gi|82602	HMW glutenin chain Dx5 - wheat	127	90524	3.7
gi|1857652	LMW glutenin storage protein [*Triticum aestivum*]	121	34340	9.7
gi|32400764	Beta-amylase [*Triticum aestivum*]	119	31100	8.9
gi|18970	D hordein [*Hordeum vulgare* subsp. *vulgare*]	57	46195	2.0
gi|32400762	AmiB [*Triticum aestivum*]	57	27143	6.8

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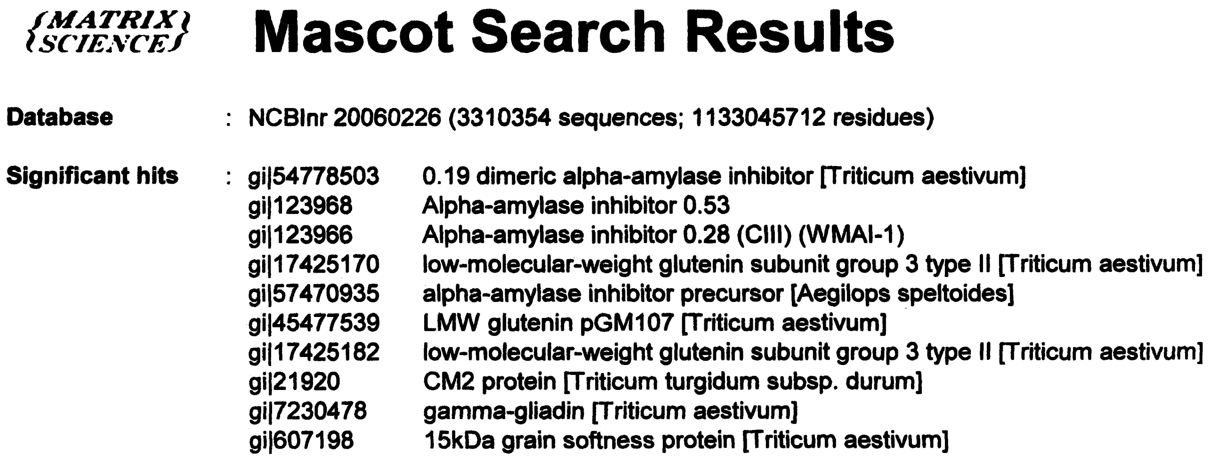
(a)



MATRIX SCIENCE

Mascot Search Results

Database : NCBInr 20060226 (3310354 sequences; 1133045712 residues)

Significant hits :

gi|54778503	0.19 dimeric alpha-amylase inhibitor [Triticum aestivum]
gi|123968	Alpha-amylase inhibitor 0.53
gi|123966	Alpha-amylase inhibitor 0.28 (CIII) (WMAI-1)
gi|17425170	low-molecular-weight glutenin subunit group 3 type II [Triticum aestivum]
gi|57470935	alpha-amylase inhibitor precursor [Aegilops speltoides]
gi|45477539	LMW glutenin pGM107 [Triticum aestivum]
gi|17425182	low-molecular-weight glutenin subunit group 3 type II [Triticum aestivum]
gi|21920	CM2 protein [Triticum turgidum subsp. durum]
gi|7230478	gamma-gliadin [Triticum aestivum]
gi|607198	15kDa grain softness protein [Triticum aestivum]

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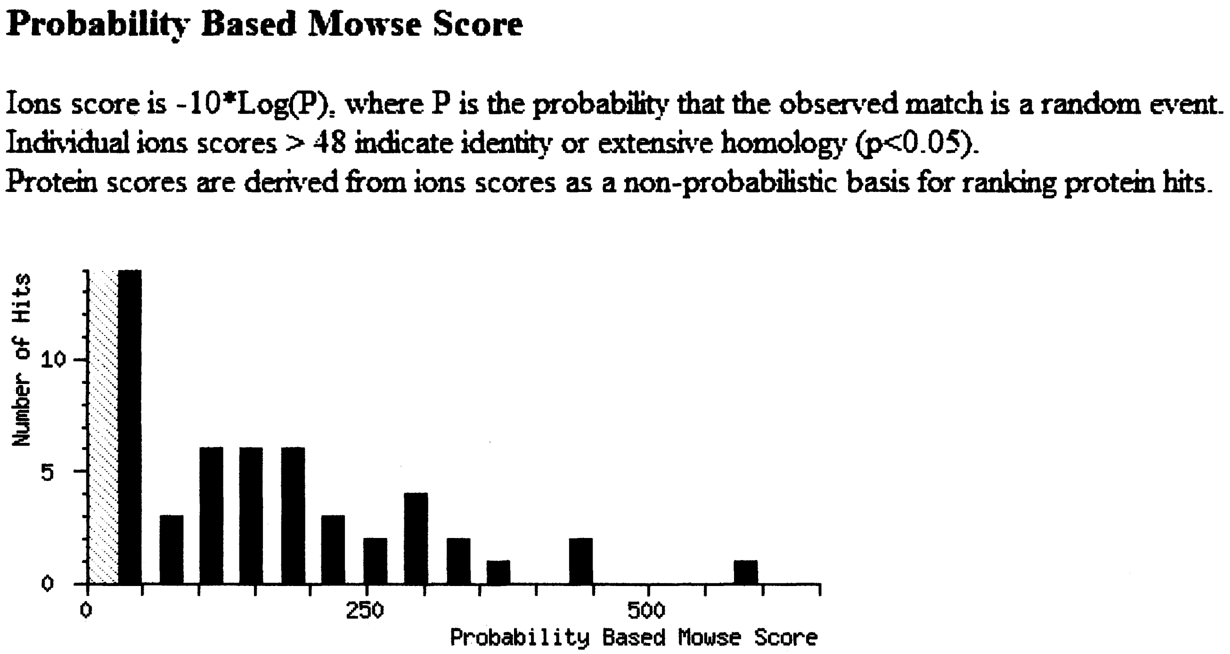
(b)



Probability Based Mowse Score

Ions score is -10*Log(P), where P is the probability that the observed match is a random event. Individual ions scores > 48 indicate identity or extensive homology ($p<0.05$).
Protein scores are derived from ions scores as a non-probabilistic basis for ranking protein hits.

Figure 1. Mascot search result for a soft wheat flour extract displayed in peptide summary format showing: (a) the top 10 significant hits, (b) the protein score distribution histogram, and (c) the detailed information for the first significant hit.

Continued on next page.

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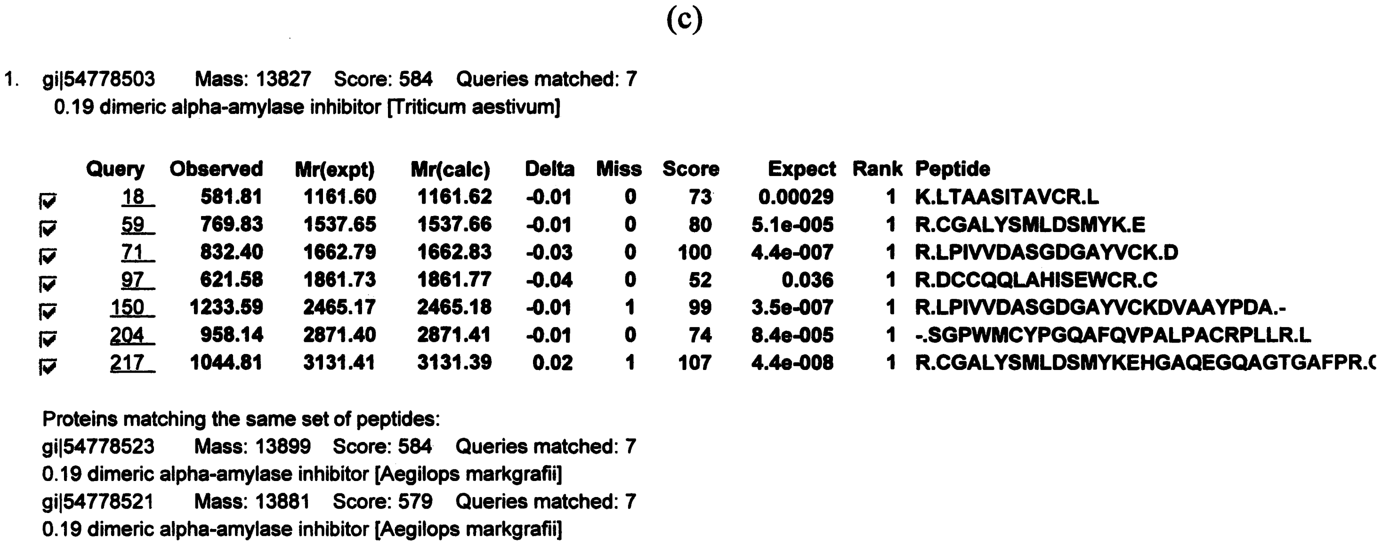

(c)

```
1.  gi|54778503      Mass: 13827   Score: 584   Queries matched: 7
     0.19 dimeric alpha-amylase inhibitor [Triticum aestivum]

      Query  Observed   Mr(expt)   Mr(calc)   Delta  Miss  Score    Expect  Rank  Peptide
☑      18      581.81    1161.60    1161.62   -0.01    0     73   0.00029    1   K.LTAASITAVCR.L
☑      59      769.83    1537.65    1537.66   -0.01    0     80   5.1e-005   1   R.CGALYSMLDSMYK.E
☑      71      832.40    1662.79    1662.83   -0.03    0    100   4.4e-007   1   R.LPIVVDASGDGAYVCK.D
☑      97      621.58    1861.73    1861.77   -0.04    0     52     0.036    1   R.DCCQQLAHISEWCR.C
☑     150     1233.59    2465.17    2465.18   -0.01    1     99   3.5e-007   1   R.LPIVVDASGDGAYVCKDVAAYPDA.-
☑     204      958.14    2871.40    2871.41   -0.01    0     74   8.4e-005   1   -.SGPWMCYPGQAFQVPALPACRPLLR.L
☑     217     1044.81    3131.41    3131.39    0.02    1    107   4.4e-008   1   R.CGALYSMLDSMYKEHGAQEGQAGTGAFPR.

Proteins matching the same set of peptides:
gi|54778523      Mass: 13899   Score: 584   Queries matched: 7
0.19 dimeric alpha-amylase inhibitor [Aegilops markgrafii]
gi|54778521      Mass: 13881   Score: 579   Queries matched: 7
0.19 dimeric alpha-amylase inhibitor [Aegilops markgrafii]
```

Figure 1. Continued.

Table IV. Protein Summary from MASCOT MS/MS Ion Search Result for the Barley Flour Extract

Protein Accession no.	*Protein Description*	*Protein Score*	*Protein Mass*	*Protein % Coverage*
gi|452325	CMd component of tetrameric alpha-amylase inhibitor [*Hordeum vulgare* subsp. *vulgare*]	523	19140	58.5
gi|3367714	BDAI-1; Barley dimeric alpha-amylase inhibitor [*Hordeum vulgare* subsp. *vulgare*]	517	17045	55.9
gi|82371	B3 hordein (clone pB7) - barley (fragment)	345	30618	25.0
gi|82548223	B hordein [*Hordeum vulgare* subsp. *vulgare*]	345	30593	24.9
gi|2392400	Beta-zein complexed with palmitoyl coenzyme A, Nmr, 16 structures	338	10145	71.4
gi|1167498	D hordein [*Hordeum vulgare* subsp. *vulgare*]	268	75633	11.2
gi|34365052	Putative gamma-2 hordein [*Hordeum vulgare*]	243	29471	19.6
gi|19009	CMe [*Hordeum vulgare* subsp. *vulgare*]	184	16695	17.6
gi|288709	Gamma-3 hordein [*Hordeum vulgare*]	182	33252	17.5
gi|9650762	Trypsin inhibitor, BTICMc [*Hordeum vulgare* subsp. *vulgare*]	175	15796	36.4
gi|82368	Alpha-amylase/trypsin inhibitor CMc - barley (fragment)	124	3408	72.4
gi|18929	B hordein precursor [*Hordeum vulgare* subsp. *vulgare*]	122	33938	10.0
gi|19003	Monomeric alpha-amylase [*Hordeum vulgare* subsp. *vulgare*]	85	16376	14.4
gi|19041	Lipid transfer protein [*Hordeum vulgare* subsp. *vulgare*]	84	10806	12.7
gi|6634471	BTI-CMe2.1 [*Hordeum vulgare* subsp. *vulgare*]	73	16825	8.8

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Table V. Protein Summary from MASCOT MS/MS Ion Search Result for the Rye Flour Extract

Protein Accession no.	*Protein Description*	*Protein Score*	*Protein Mass*	*Protein % Coverage*
gi|225729	Trypsin inhibitor	539	13809	66.9
gi|11493665	Secalin precursor [*Secale cereale*]	351	52212	11.6
gi|21713	Unnamed protein product [*Triticum aestivum*]	213	18893	19.0
gi|123968	Alpha-amylase inhibitor 0.53	113	13690	12.9
gi|21192	Alpha-D-maltose; beta-amylase [*Secale cereale*]	112	24562	12.6
gi|18970	D hordein [*Hordeum vulgare* subsp. *vulgare*]	95	46195	2.9
gi|225102	Trypsin/amylase inhibitor pUP13	79	15307	9.6
gi|20798981	WCI proteinase inhibitor [*Triticum aestivum*]	74	13506	35.3
gi|452323	CMb component of tetrameric alpha-amylase inhibitor [*Hordeum vulgare* subsp. *vulgare*]	73	17199	18.1
gi|32400764	Beta-amylase [*Triticum aestivum*]	59	31100	8.9
gi|6684164	Glutenin, high molecular weight subunit type y precursor [*Triticum aestivum*]	49	76621	1.4
gi|21202	Sec1 precursor [*Secale cereale*]	47	41515	5.0
gi|50402256	Grain softness protein [*Secale cereale*]	46	18907	8.5

Table VI. Protein Summary from MASCOT MS/MS Ion Search Result for the Oat Flour Extract

Protein Accession no.	*Protein Description*	*Protein Score*	*Protein Mass*	*Protein % Coverage*
gi|4868127	Puroindoline precursor [*Avena fatua*]	320	17591	34.7
gi|693794	Alcohol-soluble avenin-3=23.2 kDa protein [*Avena sativa*=oat, Narymsky 943, Peptide, 201 aa]	266	23718	34.3
gi|114720	Avenin precursor	249	24670	22
gi|166557	Avenin	177	25911	15.8
gi|226123	Avenin fast component N9	175	21479	19.2
gi|166568	Seed storage protein	159	24506	28.2
gi|136429	Trypsin precursor	97	25078	12.1
gi|17425174	LMW glutenin subunit group 3 type II [*Triticum aestivum*]	47	42455	4.9

proteins included are the alpha-amylase inhibitors and lipid transfer protein. The rye flour was found to have some wheat and barley proteins in addition to the rye proteins (secalin precursor, sec1 precursor and beta-amylase). Avenin, the prolamin from oat was identified in the oat flour extract. Traces of some LMW glutenin from wheat were also detected, indicating some contamination of the oat flour.

Results from the gluten-free flours – rice, corn, and buckwheat – are shown in Table VII. Prolamin from rice and zein from corn were readily identified. The buckwheat extracts had very few proteins that were found in this alcohol/water fraction.

The 7 grain flour mix was found to contain mostly proteins from wheat, including the amylases and the gliadins, and some proteins from rye and barley. Proteins from triticale (which is a hybrid of wheat and rye), corn, millet, and soy were not detected. These grains could be present in a relatively small percentage in the mix. The chromatogram obtained from this extract was very complex and is shown in Figure 2. This is a typical LC-MS/MS display of chromatograms obtained from a survey DDA acquisition mode. The bottom box showed the total ion chromatogram – base peak intensities (BPI) obtained from the MS scanning mode – while the 3 boxes above showed the three channels of MS/MS data from the run. Each line in the top 3 boxes represents an acquisition of a MS/MS spectrum from a multiple charged ion derived from a peptide detected in the MS mode. If more than 3 peptides eluted at the same time, the ones with lower intensities would not be detected. One approach to rectify this is to slow down the LC gradient. Another is to increase the column length or use a different column to improve resolution of the LC separation. Most of the chromatography was performed on the 100 mm PepMap C_{18} column which is a fused silica column packed with spherical silica particles coated with a C_{18} stationary phase. These columns have been successful with LC/MS proteomic studies involving the separation of peptides. Recently, a new type of high performance liquid chromatography column based on the porous monolithic stationary phases has been introduced. Instead of particles, the column is filled with a porous rod. There are two major types – silica based and polymer based. Onyx, the column used in this study is a silica based C_{18} column. The chemistry of this column is very similar to a particulate column, but with the advantage that the rate of mass transfer is enhanced. The eluent flow is faster, column back pressure is lower and the column takes less time to re-equilibrate. Shorter analysis time was achieved with separation efficiency similar to a particulate column. In addition, column flow can be reversed to remove particulates that collected on the inlet side of the system during use. It is anticipated that these monolithic columns will become more popular in protein and peptide LC studies.

In summary, we characterized the proteins found in the major grain flours using an LC-MS/MS approach after minimal sample work up that included extraction and enzymatic digestion. Further work based on this method will be performed to confirm glutens found in other food matrices.

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Table VII. Protein Summary from MASCOT MS/MS Ion Search Result for the Extracts from Gluten Free Flour: Rice, Corn and Buckwheat

Protein Accession no.	*Protein Description*	*Protein Score*	*Protein Mass*	*Protein % Coverage*
Rice (White)				
gi|1842176	Prolamin [*Oryza sativa* (japonica cultivar-group)]	486	16690	49.0
gi|1398913	Allergenic protein [*Oryza sativa* (japonica cultivar-group)]	262	18533	51.2
gi|34900250	13 kDa prolamin [*Oryza sativa* (japonica cultivar-group)]	151	17062	42.4
gi|311893	Major allergenic protein (RAP) [*Oryza sativa*]	103	18056	37.0
Corn				
gi|22536	Zein protein 3 [*Zea mays*]	246	29102	18.0
gi|16305123	19 kDa alpha-zein D2 [*Zea mays*]	223	26774	19.1
gi|168697	Zein	112	22887	15.9
gi|22529	Unnamed protein product [*Zea mays*]	109	26395	14.2
gi|16305121	19 kDa alpha-zein D1 [*Zea mays*]	93	26601	10.4
Buckwheat (light)				
gi|1168155	BWI-1=protease inhibitor/trypsin inhibitor [*Fagopyrum esculentum*=buckwheat plants, cv. Shatilovskay]	160	7857	43.5
gi|29839254	13S globulin seed storage protein 1 precursor (Legumin-like protein 1) [Contains: 13S globulin seed	127	64821	10.8
gi|4895075	Major allergenic storage protein [*Fagopyrum esculentum*]	124	61469	13.6

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Table VIII. Protein Summary from MASCOT MS/MS Ion Search Result for the 7 Grain Flour Extract

Protein Accession No.	*Protein Description*	*Protein Score*	*Protein Mass*	*Protein % Coverage*
gi|21713	Unnamed protein product [*Triticum aestivum*]	441	18893	52.4
gi|56480630	0.19 Dimeric alpha-amylase inhibitor [*Triticum aestivum*]	266	13752	33.1
gi|21192	Alpha-D-maltose; beta-amylase [*Secale cereale*]	157	24562	16.2
gi|10803445	Lipid transfer protein [*Triticum turgidum* subsp. *durum*]	146	10281	34.4
gi|32400764	Beta-amylase [*Triticum aestivum*]	134	31100	13.8
gi|68844	Alpha-amylase inhibitor CIII - wheat	132	13961	20.3
gi|21206	Unnamed protein product [*Secale cereale*]	117	22341	8.8
gi|121104	Gamma-gliadin precursor	112	34791	7.6
gi|15148400	Gamma-gliadin [*Triticum aestivum*]	110	24424	10.8
gi|607198	15 kDa grain softness protein [*Triticum aestivum*]	87	17670	16.1
gi|121101	Gamma-gliadin precursor	85	37498	4.0
gi|18907	Unnamed protein product [*Hordeum vulgare*]	73	21086	6.1
gi|452323	CMb component of tetrameric alpha-amylase inhibitor [*Hordeum vulgare* subsp. *vulgare*]	68	17199	13.4
gi|18980	Unnamed protein product [*Hordeum vulgare* subsp. *vulgare*]	66	35285	4.3
gi|21751	HMW glutenin subunit 10 [*Triticum aestivum*]	63	69986	1.7
gi|20798981	WCI proteinase inhibitor [*Triticum aestivum*]	62	13506	26.9
gi|121103	Gamma-gliadin B precursor	60	33402	4.5
gi|19037	Lipid transfer protein [*Hordeum vulgare* subsp. *vulgare*]	57	12749	13.7
gi|18914	Unnamed protein product [*Hordeum vulgare*]	56	30632	3.4

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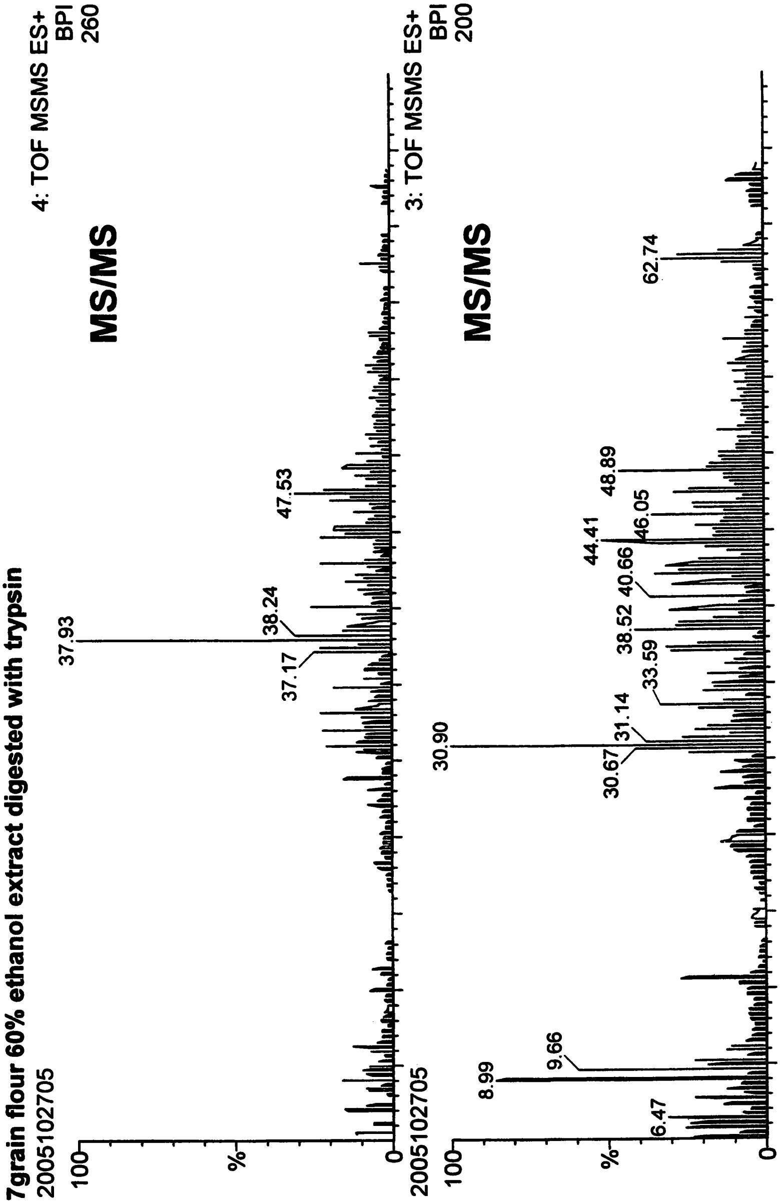

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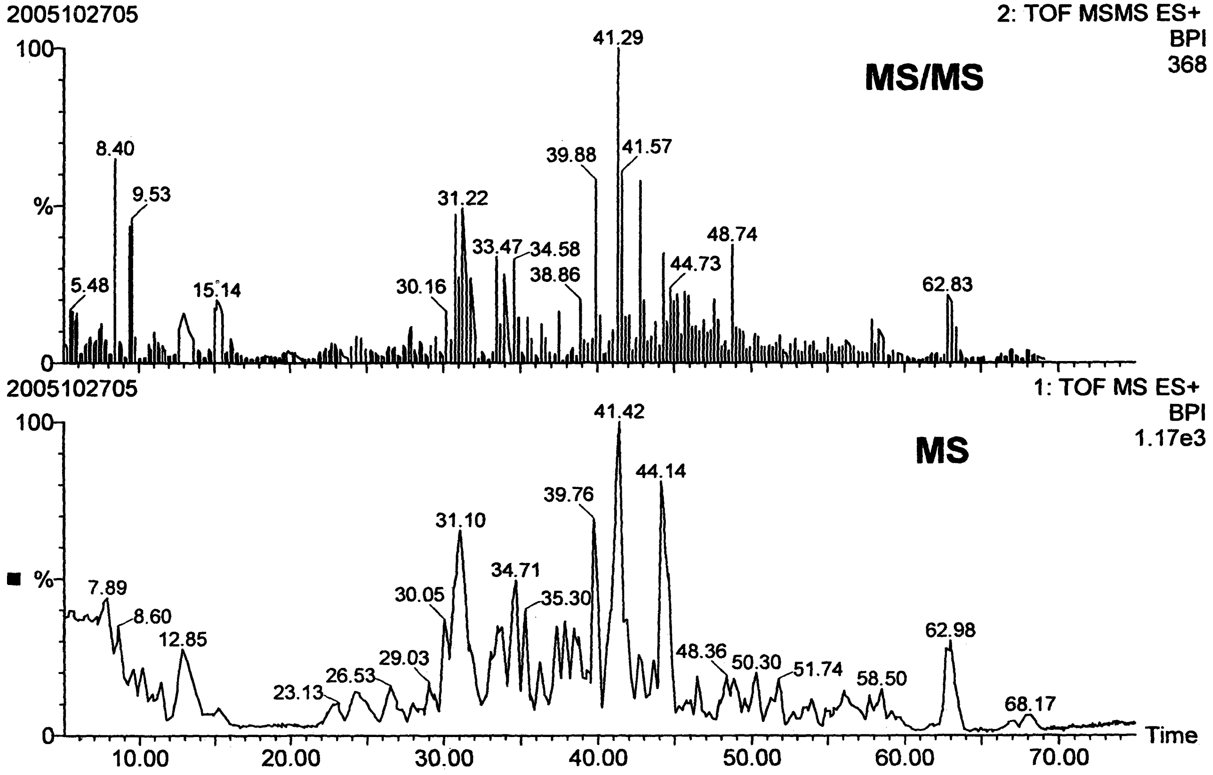

Figure 2. Raw data display of the 7 grain flour extract showing the MS scan (bottom trace) and three channels of MS/MS data (top three traces) from a survey DDA acquisition.

Conclusion

We have shown that it is possible to speciate gluten from various grains using LC-MS/MS after digestion of the alcohol fraction of the flour extract. Depending on the type of food matrices, this method can be used to identify the origin of the gluten for a sample that has tested positive using non-species-specific ELISA test kits.

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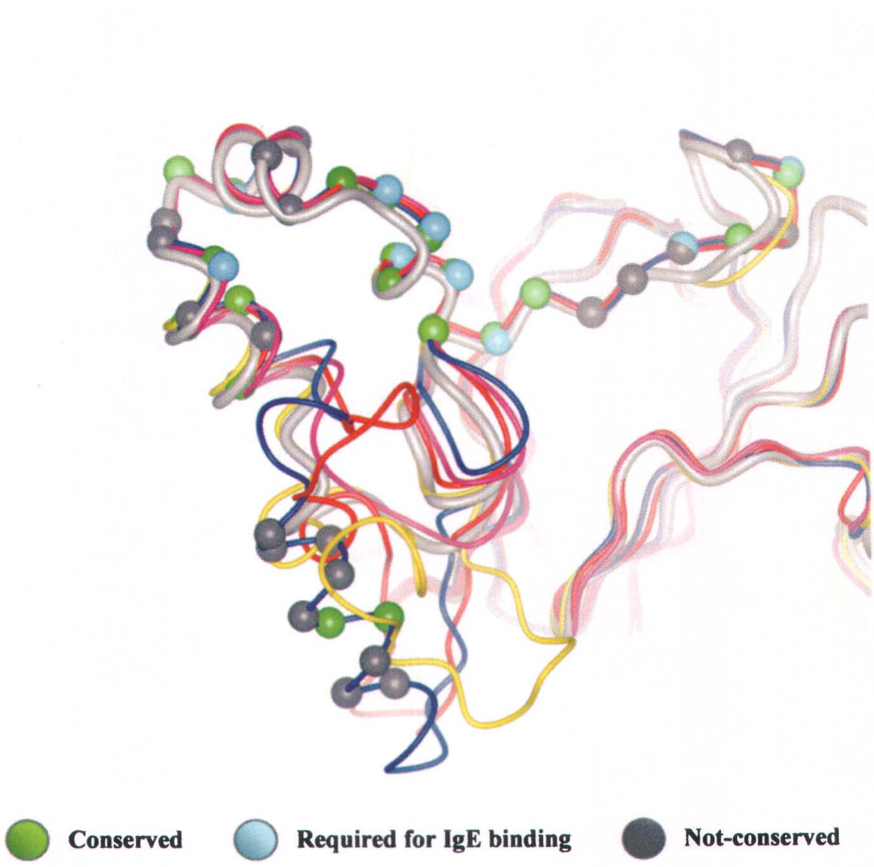

Figure 20.5. Backbone representation of Ara h 1 homology models generated from SWISS-MODEL (yellow), 3D-JIGSAW (blue) and Phyre (magenta) superposed upon the 2PHL X-ray crystal structure template from kidney bean (gray). Conserved residues are represented by green spheres located at corresponding backbone alpha-carbons. Those necessary for serum IgE binding are represented by cyan spheres. Nonconserved residues are represented by gray spheres. The location of IgE epitopes 10-13 are indicated (17).

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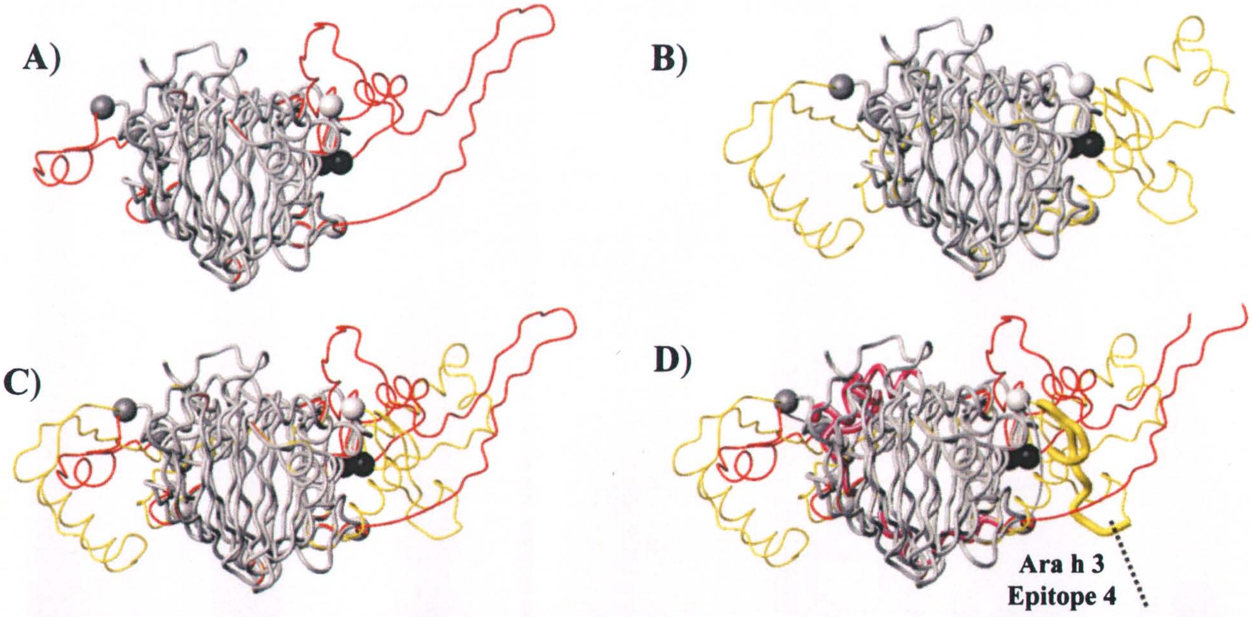

Figure 20.9. A) Superposition of the SWISS-MODEL soybean proglycinin model structure (red) and the 1FXZ X-ray soybean proglycinin structure template (21). Submission of the complete soybean proglycinin amino acid sequence to the SWISS-MODEL homology modeling server was done in an effort to obtain a more complete structure for comparison to homology models generated for Ara h 3. B) Ara h 3 homology model generated from SWISS-MODEL (yellow) superimposed upon the 1FXZ X-ray soybean proglycinin crystal structure template (gray). C) Superposition of the Ara h 3 homology model generated from SWISS-MODEL (yellow), the SWISS-MODEL soybean proglycinin model structure (red) and the 1FXZ X-ray crystal structure template (gray). D) Annotation of the overlaid SWISS-MODEL structures with the location of Ara h 3 allergenic epitopes 1-4 (16). The relatively sequence-similar epitopes 1-3 are highlighted as thicker backbone magenta representations while the sequence-dissimilar epitope 4 is highlighted in yellow.

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